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The Metabolism and Antiketogenic Effects of Sorbitol. Sorbitol Dehydrogenase

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(*Received* 23 *November* 1950)

Using the perfused liver of fasted or phlorrhizinized dogs, Embden & Griesbach (1914) discovered that sorbitol was converted into fermentable reducing sugars from which the only identifiable osazone prepared was phenylglucosazone. On the basis of changes in optical rotation and in the Seliwanoff reaction during perfusion, these authors considered that the primary product of sorbitol oxidation was fructose which was subsequently transformed into glucose. Additional evidence consistent with this view was provided by Anschel (1930), who observed a large increase in the excretion of fructose following administration of sorbitol to a case of fructosuria.

The work of Embden & Griesbach prompted the suggestion that sorbitol might prove a useful carbohydrate source in diabetes mellitus (Thannhauser & Meyer, 1929); but attempts to assess the utilization of sorbitol in diabetics by measurement of respiratory quotients and blood-sugar levels led to much controversy. The evidence has been reviewed by Carr & Krantz (1945). The effect of feeding sorbitol upon the deposition of glycogen in rat liver has also been the subject of conflicting reports. Nevertheless, most workers agree that sorbitol can act as a glycogen precursor (Carr & Forman, 1939; Johnston & Deuel, 1943; Blatherwick, Bradshaw, Ewing, Larson & Sawyer, 1940). Some insight into the enzymic mechanism of these metabolic reactions was gained by Breusch (1942, 1943), who demonstrated sorbitol dehydrogenase activity in brei prepared from the livers of starved cats. The criteria employed were the reduction of oxaloacetate and of methylene blue.

The work described in this paper was undertaken with the object of obtaining more precise definition of the initial steps in sorbitol metabolism. Such information is particularly desirable in view of the antiketogenic property of sorbitol, described by Edson (1936), who found that sorbitol is more effective than glucose or fructose in suppressing spontaneous ketogenesis in the liver slices of starved rats.

EXPERIMENTAL

Rats of the Wistar strain, aged 8–12 months, were used. They were fed a stock diet of pollard, bran, maize meal, meat meal and bone flour. In starvation experiments food was completely withheld for 22–36 hr. but free access to water allowed.

Tissue slices were cut by the conventional freehand method. Approx. 15 mg. dry weight of slices per Warburg flask were used.

Homogenates were prepared with the all-glass Potter-Elvehjem apparatus. The organs were removed immediately after the animals had been killed by a blow on the head or, in the case of rats, by rupturing the spinal cord, and chilled in ice water. After homogenizing, the material was kept at 0° until used.

Manometric methods. The standard methods of Warburg were used for the measurement of O_2 consumption by slices, homogenates and other preparations. Slices were suspended in phosphate saline, pH 7·4 (Krebs, 1933). Substrates dissolved in a small volume of distilled water were added to the main compartment of Warburg vessels; in controls with substrate omitted an equal volume of distilled water was added. Acid substrates were added as neutral Na salts; amines were added as neutralized hydrochlorides. Flasks were equilibrated in the thermostat for 7–10 min. before manometric readings were commenced.

Analytical methods

Sorbitol was determined by the periodate method of Rappoport, Reifer & Weinmann (1937).

Reducing sugar was determined according to Somogyi (1945*a*) after deproteinizing with $Ba(OH)_2$-$ZnSO_4$ (Somogyi, 1945*b*). When experiments were performed in the presence of methylene blue, the dye was removed from the deproteinized filtrate by treatment with a minimum amount of Merck's medicinal charcoal. Reducing sugar was determined in the solution (Somogyi, 1945*a*) after removal of the charcoal by filtration.

Ketose was determined by Cole's modification of Roe's method (see Bacon & Bell, 1948). When necessary the solution was cleared of methylene blue as above. The colour intensity was estimated by measuring optical density at 483 mμ. In estimations of fructose or sorbose the colour was always compared with that developed in a standard solution of the hexose in question, because sorbose gives approximately half the colour of an equal concentration of fructose.

Glucose was determined manometrically using glucose oxidase (Keilin & Hartree, 1948) in the presence of catalase (solution of crystallized enzyme), but without the addition of ethanol which raised the blank O_2 uptake to an unduly large and variable value.

Glucose oxidase was prepared from *Aspergillus niger* (National Collection of Type Cultures, no. 594), a culture of which was kindly supplied by Dr J. S. D. Bacon. The mould was grown as described by Mann (1944), harvested on the 4th day of incubation, and a dry powder prepared from the mycelium by homogenizing in a Waring blender with cold acetone. The acetone-dried powder was extracted with

0·03 M-potassium phosphate buffer, pH 6, the yellow filtrate treated with 7 vol. of cold acetone and the mixture immediately centrifuged in order to separate the viscous precipitate. The vacuum-dried precipitate was stable at 2°. When required, it was dissolved in 0·2 M-potassium phosphate buffer, pH 6, and dialysed against 0·01 M-potassium phosphate buffer, pH 6. This solution had high glucose oxidase activity, but did not oxidize galactose, fructose, sorbose, mannose or sorbitol.

Acetoacetic acid was determined by the aniline citrate method (Edson, 1935) except when determined simultaneously with β-hydroxybutyric acid when the modified Van Slyke technique (Edson, 1935) was employed.

Lactic acid was determined by the method of Friedemann & Graeser (1933).

Nitrogen was determined by the titrimetric micro-Kjeldahl procedure of Hiller, Plazin & Van Slyke (1948).

Coenzyme I (CoI) was estimated by the method of LePage (1947), using the value $E^{1\%}_{1\,cm.} = 8500$ at 340 mμ. In enzymic reactions the reduced coenzyme was estimated from the increase in optical density at 340 mμ. above the value of the blank at zero time.

Hexokinase activity was estimated according to Colowick & Kalckar (1943), aldolase activity according to Taylor, Green & Cori (1948).

pH *Measurements* were made with a glass electrode.

Optical density was measured in a Beckman quartz spectrophotometer, DU model.

Materials

Sorbitol. A sample of D-sorbitol monohydrate obtained from the Pfanstiehl Chemical Co. was used throughout this work. $[\alpha]_D^{23°} = -1·73 \pm 0·05°$ in water (*c*, 9·1).

Acetoacetate was prepared as described by Ljunggren (1924).

Cytochrome c was prepared by the method of Keilin & Hartree (1937) and assayed according to Potter (1945).

Coenzyme I. A crude preparation (12% purity) was made from baker's yeast by the method of Williamson & Green (1940). Some of this material was treated with norit according to Clark, Dounce & Stotz (1949), and the purity raised to 25%. Cozymase (purity 34 and 60%) obtained from Schwartz Laboratories, Inc., New York, was used in some experiments.

Reduced coenzyme I ($CoIH_2$) was prepared by the method of Ohlmeyer (1938).

Metabolic units

$-Q_{O_2}$ = μl. O_2 consumed/mg. dry wt. of tissue/hr.

Q_{Keto} = μl. CO_2 equivalent to β-ketonic acid formed/mg. dry wt. of tissue/hr.; 1 μmoi. β-ketonic acid ≡ 22·4 μl. CO_2.

$Q_{Hydroxy}$ = μl. CO_2 equivalent to β-hydroxybutyric acid formed/mg. dry wt. of tissue/hr.; 1 μmol. β-hydroxybutyric acid ≡ 22·4 μl. CO_2.

RESULTS

Oxidation and antiketogenic effect of sorbitol in liver slices

Sorbitol is rapidly oxidized by rat-liver slices whether the animals have been well fed or starved. This is indicated by the increase in oxygen consumption observed when sorbitol is present in the suspension medium (Table 1). In agreement with Edson (1936) it was consistently found that 0·01 M-sorbitol caused a marked decrease (25–50%) in acetoacetate formed endogenously by liver slices from fasted rats. Since this observation does not exclude the possibility of acetoacetate being reduced to β-hydroxybutyrate in the presence of sorbitol, both ketone bodies were determined in presence and absence of sorbitol. The results show that formation of β-hydroxybutyrate was decreased to about the

Table 1. *Oxidation and antiketogenesis in rat-liver slices*

(Slices incubated in conical Warburg vessels containing 3·00 ml. of phosphate saline, pH 7·4. Inseals contained 0·20 ml. 2 N-NaOH. Time, 2 hr. Gas, O_2. Temp. 38°.)

Exp. no.	State of animal	Substrate added (0·01 M)	$-Q_{O_2}$	Q_{Keto}
1	Well fed	Nil	11·9	1·02
		Pentane-1-carboxylate	16·1	3·78
		Pentane-1-carboxylate + sorbitol	17·0	4·22
2	Fasted 36 hr.	Nil	11·0	1·93
		Sorbitol	15·8	0·83
		Glucose	11·7	1·73
		Fructose	13·9	1·29
		Sorbose	9·8	1·04
		L-Arabinose	11·7	1·62
		D-Arabinose	11·8	1·41
3	Fasted 36 hr.	Nil	13·8	3·82
		Glucose-1-phosphate (K salt)	13·5	3·33
4	Fasted 24 hr.	Nil	10·4	2·33
		Sorbitol	12·7	0·89
		Malonate (0·01 M)	10·3	2·68
		Sorbitol + malonate (0·01 M)	9·6	1·47
5	Fasted 24 hr.	Nil	11·8	3·44
		Sorbitol	15·4	1·89

In Exp. 5, $Q_{Hydroxy}$ was 0·96 in absence of added substrate, 0·50 in presence of sorbitol.

same extent as acetoacetate production in the presence of 0·01 M-sorbitol (Table 1, Exp. 5). Thus sorbitol decreased the total ketone bodies formed spontaneously in liver slices of starving rats.

Although sorbitol reduces the spontaneous ketogenesis of liver slices considerably, this compound has little effect on the production of ketone bodies during oxidation of pentane-1-carboxylic acid added to liver slices (Table 1). None of the carbohydrates tested exerted an effect on spontaneous ketogenesis as great as that of sorbitol (Table 1). Likely products of sorbitol oxidation (glucose, fructose and sorbose) possessed weaker antiketogenic properties when tested in a concentration of 0·01 M. The fact that neither L- nor D-arabinose exerted an appreciable antiketogenic effect has special interest, since Breusch (1943) reported that cat liver exhibits dehydrogenase activity for D-arabinose.

The antiketogenic effect of sorbitol is capable of several interpretations: it could operate (1) by inhibiting the oxidation of ketone-body precursors such as fatty acids, pyruvate and certain amino-acids, (2) by diverting the oxidation of fatty acids and pyruvate through pathways which do not lead to acetoacetate, or (3) by oxidative removal of the ketone bodies formed. There is evidence to indicate that in the liver complete oxidation via the Krebs tricarboxylic acid cycle is the main alternative to ketone-body formation (Lehninger, 1946; Grafflin & Green, 1948). Consequently, the effect of Krebs-cycle inhibitors, such as malonate, on antiketogenesis due to sorbitol possesses some significance.

The effect of malonate was tested by incubating liver slices from fasted rats with malonate in presence and absence of sorbitol. The results indicate that 0·01 M-malonate caused an increase in the amount of acetoacetate formed both in presence and absence of sorbitol, but by no means abolished the antiketogenic action (Table 1). Consequently this experiment affords no evidence that the Krebs cycle is directly concerned in the antiketogenic effect of sorbitol.

Products of the oxidation of sorbitol by liver slices

During the oxidation of sorbitol by liver slices of fasted rats, there appeared in the medium an extra amount of reducing sugar (calculated as glucose) approximately equivalent to the sorbitol disappearing; but when liver slices of well fed rats were used the extra reducing sugar formed was only slightly in excess of that appearing in the control without sorbitol (Table 2).

In experiments on a larger scale sorbitol was oxidized in presence of liver slices, and the suspension fluid analysed for total reducing sugar, glucose (using glucose oxidase) and ketose. Table 3 shows that sorbitol was largely converted to glucose, but a small amount of ketose was invariably produced. The amount of lactic acid formed was very small (0·07 mg./ml. of suspension medium). The

Table 2. *Formation of reducing sugar during sorbitol oxidation by rat-liver slices*

(Conditions as in Table 1.)

Exp. no.	State of animal	Substrate	$-Q_{O_2}$	Reducing sugar (calc. as glucose) (μmol./mg. dry wt.)	Sorbitol disappearing (μmol./mg. dry wt.)
1	Well fed	Nil	10·8	1·28	0
		Sorbitol (0·01 M)	14·8	1·33	0·66
2	Well fed	Nil	11·5	1·00	0
		Sorbitol (0·02 M)	10·6	1·24	0·72
3	Fasted 22 hr.	Nil	12·9	0·09	0
		Sorbitol (0·01 M)	15·2	0·66	0·68
4	Fasted 24 hr.	Nil	11·2	0·07	—
		Sorbitol (0·01 M)	12·1	0·50	—

Table 3. *Products of the oxidation of sorbitol by liver slices*

(Slices from male rats, fasted 36 hr., suspended in 12·0 ml. of phosphate saline, pH 7·4, in 150 ml. Erlenmeyer flasks. Sorbitol concentration, 0·01 M. Time, 4 hr. Gas, O_2. Temp. 38°. Suspension fluid deproteinized with $Ba(OH)_2$-$ZnSO_4$ before determination of optical rotation and analysis. For determination of reducing sugar, glucose and ketose see Analytical methods section.)

Rat no.	Dry wt. of slices (mg.)	Sorbitol added (mg.)	$\alpha_D(l, 4)$	Reducing sugar (calc. as glucose) (mg.)	Glucose (mg.)	Ketose (calc. as fructose) (mg.)
1	104·8	Nil	—	4·0	—	—
	109·3	21·6	+0·05°	19·0	18·0	0·24
2	102·9	Nil	—	2·4	—	—
	119·0	21·6	+0·05°	18·0	16·5	0·24
3	93·3	Nil	—	3·8	—	—
	123·0	21·6	+0·05°	20·8	17·7	0·26

values of the optical rotation are consistent with the observed concentrations of reducing sugars. The reducing material reacted with phenylhydrazine under the conditions specified by Edson (1940) to give a crystalline compound with the typical appearance of phenylglucosazone accompanied by a small amount of amorphous material. The reducing sugar was completely fermented by a suspension of washed baker's yeast. In experiments similar to those in Table 3 it was found that no ketose was formed in absence of sorbitol, and the deproteinized filtrate had an optical rotation of $-0{\cdot}01 \pm 0{\cdot}01°$ (*l*, 4).

Embden & Griesbach (1914) obtained evidence that during the oxidation of sorbitol by perfused dog liver, fructose was the primary product, but was subsequently converted to glucose. Confirmation of such a sequence was sought in rat liver, sorbitol being incubated with slices for varying periods of time, and the suspension medium analysed for ketose and total reducing sugar (Table 4). Although

genized in ice-cold 0·88M-sucrose. All subsequent operations were carried out in the cold room (2°). Nuclei, whole cells and debris were removed by centrifuging at 600*g* for 10 min. The supernatant (fraction 1) was decanted and recentrifuged twice in the same way. In order to remove mitochondria, 0·11 vol. of 1·5M-sodium chloride was added and the mixture kept for 5 min. in an ice bath. The agglutinated mitochondria were centrifuged at 2400*g* for 10 min., and the sediment (fraction 2) resuspended in 0·15M-sodium chloride. The whole homogenate and the several fractions were then tested for their ability to oxidize sorbitol by measuring oxygen consumption (Table 6).

Most of the active material remained in the supernatant (fraction 1) obtained after removal of nuclei. Neither the mitochondria (fraction 2) nor the final supernatant (fraction 3) consumed much oxygen, but recombination of supernatant and mitochondria restored sorbitol oxidation to the initial level. The

Table 4. *Production of ketose during sorbitol oxidation by liver slices*

(Rat fasted 36 hr. Slices incubated in conical Warburg vessels in 3·10 ml. phosphate saline, pH 7·4. Inseals contained 0·20 ml. 2N-NaOH. Gas, O_2. Temp. 38°.)

Sorbitol (0·01M)	Time (hr.)	Reducing sugar (calc. as glucose) (μg./mg. dry wt.)	Ketose (calc. as fructose) (μg./mg. dry wt.)
Absent	0·5	5·0	0·00
Present		44·0	2·20
Absent	1·0	5·5	0·00
Present		74·5	4·35
Absent	1·5	3·5	0·00
Present		111·5	3·25
Absent	2·0	0·5	0·00
Present		153·0	5·85
Absent	2·5	10·0	0·00
Present		167·0	6·75

a significant concentration of ketose was found in all cases, no initially high proportion of ketose could be detected, the ketose concentration rising parallel to that of total reducing sugar.

Oxidation of sorbitol by homogenates of rat liver

Homogenates of rat liver, prepared in water or in 0·01M-potassium phosphate buffer, pH 7·6, exhibited an increased oxygen uptake in the presence of sorbitol. Oxidation of sorbitol by homogenates required the presence of CoI and was considerably enhanced by the addition of nicotinamide and cytochrome *c*. With such a preparation, in which the cytochrome system was responsible for electron transfer to molecular oxygen, addition of potassium chloride also caused a slight increase of oxygen uptake in presence of sorbitol. The optimum concentration for each of these components is shown in Table 5.

In order to determine the intracellular localization of the enzyme oxidizing sorbitol, rat liver was homo-

most probable explanation of this result is that a sorbitol dehydrogenase is located chiefly in fraction 3 (Table 6), which is composed of submicroscopic particles and soluble material. Since cytochrome oxidase and other insoluble components of the cytochrome system are largely confined to the mitochondria (Hogeboom, Claude & Hotchkiss, 1946), the system which causes oxygen uptake in the presence of sorbitol is not complete until the mitochondria and an adequate concentration of cytochrome *c* have been added. This hypothesis is supported by determinations of oxygen uptake in which methylene blue was substituted for the cytochrome system. Under these conditions fraction 3 showed a high oxygen uptake in the presence of sorbitol without addition of the mitochondrial fraction. In all subsequent experiments methylene blue was used to link the sorbitol dehydrogenase system to molecular oxygen.

The ability of whole homogenates to oxidize sorbitol decreased rapidly even at 0°, but the final

Table 5. *Optimum concentrations of components required for oxidation of sorbitol by rat-liver homogenates*

(Enzyme: 0·50 ml. of a 10% water homogenate of rat liver, centrifuged at 400 *g* for 10 min. to remove cell debris and nuclei. In each series the concentration of a single added component was varied, the concentrations of all other added components being maintained at the optimum level indicated by an asterisk. Total vol., 3·00 ml. Inseals contained 0·20 ml. 2N-NaOH. Time, 20 min. Gas, air. Temp. 38°.)

Component varied ...	Nicotinamide		Co I (12% purity)		Cytochrome *c*	
	Concn. (M)	O_2 uptake (μl.)	Concn. ($\times 10^{-4}$ M)	O_2 uptake (μl.)	Concn. ($\times 10^{-4}$ M)	O_2 uptake (μl.)
	0·0000	38	0·00	7	0·000	24
	0·0017	59	0·30	24	0·161	40
	0·0033	59	0·60	41	0·322	45
	0·0067	65	0·90	44	0·644*	53
	0·0100*	64	1·20*	46	0·966	47
	0·0150	62	1·80	53	1·449	46

Component varied ...	Sorbitol		KCl		Potassium phosphate, pH 7·6	
	Concn. (M)	O_2 uptake (μl.)	Concn. (M)	O_2 uptake (μl.)	Concn. (M)	O_2 uptake (μl.)
	0·0000	15	0·000	35	0·0000	50
	0·0033	36	0·033	36	0·0017	47
	0·0067	42	0·067	42	0·0033	45
	0·0100	43	0·100*	42	0·0050	55
	0·0133*	51	0·150	40	0·0067*	51
	0·0267	52	0·200	48	0·0100	51

* Concentration selected as optimum.

Table 6. *Distribution of sorbitol dehydrogenase in fractions of rat-liver homogenate*

(Enzyme: 0·50 ml. of homogenate or homogenate fraction. Each Warburg vessel contained in addition 0·30 ml. of KCl (0·10 M)*, 0·20 ml. potassium phosphate, pH 7·6 (0·067 M), 0·30 ml. nicotinamide (0·01 M), 0·20 ml. cytochrome *c* ($0{\cdot}644 \times 10^{-4}$ M), 0·20 ml. sorbitol (0·013 M, replaced by water in controls), 0·20 ml. Co I (12% purity; $1{\cdot}20 \times 10^{-4}$ M) and water to give a final vol. of 3·00 ml. Inseals contained 0·20 ml. 2N-NaOH. Time, 1 hr. Gas, air. Temp. 38°.)

Homogenate fraction	Sorbitol	O_2 uptake (μl.)	Extra O_2 uptake (μl.)
Whole homogenate	Absent	53	93
	Present	146	
Fraction 1: supernatant from nuclei	Absent	55	79
	Present	134	
Fraction 2: suspension of mitochondria	Absent	30	12
	Present	42	
Fraction 3: supernatant from mitochondria	Absent	35	29
	Present	64	
Fraction 2 + fraction 3	Absent	38	78
	Present	116	

* The figures in parentheses indicate the concentration of the components in the complete reaction medium. This notation is used in Tables 6–17 and Figs. 1–6.

supernatant from the homogenate (fraction 3) retains its activity unchanged for 24 hr. at 5°, and loses only 20% during incubation for 1 hr. at 38° (pH 7·8).

Extraction and partial purification of sorbitol dehydrogenase

The livers of three or four rats were rapidly removed, chilled in ice water and freed from remnants of diaphragm and mesentery. After cooling for several minutes the livers were quickly blotted dry, weighed and homogenized for 1·5–2 min. in 3 vol. of ice-cold 0·01 M-potassium phosphate buffer, pH 7·8, by means of a previously chilled Waring blender. The extract was rapidly cooled to 0° by vigorous stirring in a beaker placed in ethanol at −30°. In subsequent steps the temperature of the extract was kept between 0 and 5°. The pH was next adjusted to 4·7 by dropwise addition of 2N-HCl with vigorous stirring. After centrifuging in the cold room (2°) at 1100 *g* for 15 min. a red, slightly turbid supernatant (1, Table 7) of high activity was obtained. Several methods were used to fractionate this extract.

Preparation A. In earlier experiments the extract was adjusted to pH 4·0 by dropwise addition of 0·4N-HCl, and then to pH 7·8 by dropwise addition of 5% (w/v) KOH.

Table 7. *Purification of sorbitol dehydrogenase*

(Enzyme preparation, 0·25 or 0·50 ml., adjusted to pH 7·8 and tested as soon as prepared. Flasks contained in addition 0·25 ml. sorbitol (0·017 M, replaced by water in controls), 0·50 ml. potassium phosphate, pH 7·9 (0·033 M) and water to give a final vol. of 3·00 ml. Methylene blue (0·20 ml.; 0·001 M) and 0·50 ml. Co I (12% purity, $3·0 \times 10^{-4}$ M) were added from the side arm after equilibration. Inseals contained 0·20 ml. 2 N-NaOH. Gas, air. Temp. 38°. Values of O_2 uptake based on first 10 min. period, and corrected for uptakes in absence of substrate.)

Exp. no.	Enzyme preparation	Volume (ml.)	O_2 uptake (μl./hr.) Per mg. N	Per mg. dry wt.	Per ml. of solution	Total
1	Whole homogenate	130·8	—	6·4	456	59,400
	Whole homogenate adjusted to pH 4·7; ppt. discarded. Supernatant 1	95	—	11·3	300	29,400
	Supernatant 1 adjusted to pH 4·0 and then to pH 7·8. Ppt. discarded. Supernatant 2	84	—	10·1	240	22,200
	Supernatant 2 treated with ethanol-$CHCl_3$. Ppt. discarded. Supernatant 3	90	—	43·1	168	17,400
2	Supernatant 1	80	171	20·5	396	31,800
	Supernatant 2	83	154	17·3	300	24,900
	Supernatant 3	117	422	51·8	228	26,400
	Supernatant 3 twice fractionated with $(NH_4)_2SO_4$; dialysed	25	—	50·0	300	7,800

There was no precipitate at pH 4, but after this treatment a precipitate appeared on readjustment of the pH to 7·8. All subsequent steps were performed in the cold room at 2°. After removal of the precipitate (discarded) by centrifugation at 1100 *g* for 10 min., solid $(NH_4)_2SO_4$ was added to the supernatant (2, Table 7) to give a concentration of 2·15 M. The precipitate which formed was centrifuged down and discarded, and the concentration of $(NH_4)_2SO_4$ in the supernatant brought to 3·2 M by addition of the solid.

After centrifuging again at 1100 *g* for 30 min., the supernatant (discarded) was decanted carefully in order to conserve the light precipitate, which was dissolved in the minimum volume of 0·01 M-potassium phosphate, pH 7·6. After thorough dialysis against distilled water at 2°, and removal by centrifugation of the small precipitate which sometimes formed, the solution had an O_2 uptake of 240 μl. O_2/mg. N/hr. when tested under the conditions described in Table 8. The solution, which was coloured by haemoglobin and a little cytochrome *c*, had a very low O_2 uptake in absence of substrate.

Preparation B. Later it was found that a preparation of greater activity could be obtained by the use of ethanol and $CHCl_3$. After preliminary adjustment of the pH of the extract first to 4·0 and then to 7·8, followed by centrifuging to remove the precipitate which formed, the supernatant (2, Table 7) was mixed with 0·25 vol. of 95% (v/v) ethanol and 0·25 vol. of $CHCl_3$ added. The mixture was shaken vigorously in a stoppered vessel at room temperature for 1 min. and immediately returned to the cold room for centrifugation at 1100 *g* for 15–30 min. The denatured haemoglobin, cytochrome and other proteins formed a solid layer at the $CHCl_3$-water interface from which the supernatant aqueous layer was pipetted. The solution was thoroughly dialysed against distilled water at 2° to give an almost clear, yellowish-brown solution (3) the O_2 uptake of which under standard conditions (Table 7) was 420 μl. O_2/mg. N/hr. This preparation also possessed negligible O_2 uptake in absence of substrate.

Attempts to increase the activity of preparation *B* by fractionation with ammonium sulphate or ethanol were unsuccessful, although by this means coloured substances were separated from the active material. From Table 7 it may be seen that adjustment of the original extract to pH 4·0 and then to 7·8, followed by centrifugation, caused no increase in activity. However, when this step was omitted from the procedure, the activity of the final preparation was appreciably lower.

Oxidation of D-*sorbitol by dehydrogenase preparations*

Enzyme preparations made by the above procedures oxidize D-sorbitol rapidly in the presence of Co I, either aerobically using methylene blue for hydrogen transport to molecular oxygen, or anaerobically using methylene blue as hydrogen acceptor. Table 8 shows the anaerobic oxidation of sorbitol by the enzyme, and Figs. 1 and 2 show the effect of varying the concentration of methylene blue and Co I, respectively, on the oxygen uptake in aerobic experiments. Since Co II was not available, it was not possible to test the coenzyme specificity of the dehydrogenase.

Table 8. *Anaerobic oxidation of sorbitol by the dehydrogenase*

(Thunberg tubes contained 0·25 ml. Co I (12% purity, $1·5 \times 10^{-4}$ M), 0·25 ml. sorbitol (0·017 M), 1·00 ml. potassium phosphate, pH 8·0 (0·067 M), 0·20 ml. of indicator (approx. 10^{-4} M), and water to give a final vol. of 3·00 ml. Enzyme (0·50 ml. of preparation *B*) added from stopper after evacuation. Temp. 38°.)

	Indicator	Reduction time
Complete system	Methylene blue	18 min.
Co I omitted	Methylene blue	>5 hr.
Sorbitol omitted	Methylene blue	>5 hr.
Complete system	Janus green	Partial reduction (rose)
Complete system	Benzyl viologen	Partial reduction

Soluble diaphorase prepared from pig heart according to Straub (1939) caused a 27 % increase in oxygen consumption of the enzyme (preparation *B*)

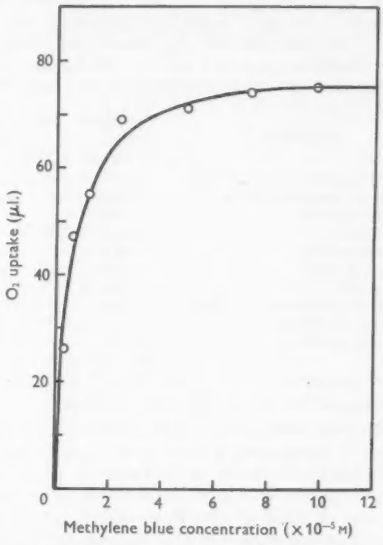


Fig. 1. Effect of methylene blue concentration on sorbitol oxidation. Flasks contained 0·30 ml. enzyme (preparation *A*), 0·25 ml. sorbitol (0·017 M), 1·00 ml. potassium phosphate, pH 8·0 (0·067 M), 0·25 ml. Co I (34 % purity, $0{\cdot}85 \times 10^{-4}$ M), methylene blue (added from side arm after equilibration) and water to give a final vol. of 3·00 ml. Gas, air. Temp. 38°. Time, 15 min.

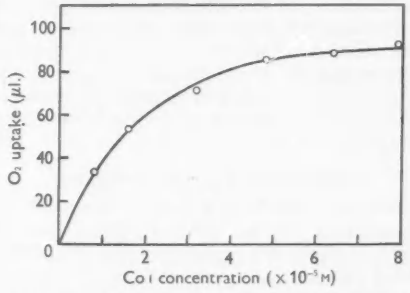


Fig. 2. Effect of Co I concentration on sorbitol oxidation. Conditions as in Fig. 1. Methylene blue concentration 10^{-3} M.

when methylene blue and Co I were present in optimum concentrations. This indicates that as in other dehydrogenase systems, diaphorase accelerates the rate of oxidation with methylene blue.

Effect of temperature. This is shown in Fig. 3. The optimum temperature under the particular conditions was 38–40°, and the activity declined steeply outside this range.

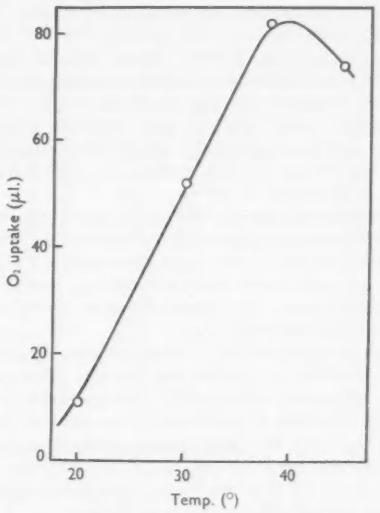


Fig. 3. Effect of temperature on sorbitol dehydrogenase activity. Conditions as in Fig. 1. Methylene blue concentration 10^{-3} M. Time, 30 min.

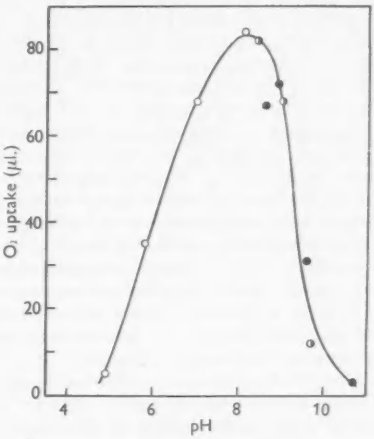


Fig. 4. Effect of pH on sorbitol dehydrogenase activity. Conditions as in Fig. 1 except that various buffers (0·067 M) were used. Time, 30 min. ○—○, Phosphate; ◑—◑, glycine-NaCl; ●—●, veronal.

Effect of pH. Fig. 4 illustrates the relationship of pH to enzymic activity, the optimum range being pH 7·9–8·1. This was confirmed by measuring the rate of Co I H_2 formation during oxidation of sorbitol

in absence of methylene blue. The enzyme appears to lose little activity within the pH range 5–9 when incubated at 38° for 40 min. in absence of substrate. At pH 3·2 activity is completely destroyed in a few seconds at 0°.

Enzyme-substrate affinity. From data on the influence of sorbitol concentration on the initial rate of oxygen uptake by the enzyme at 38° in presence of phosphate buffer (pH 8·0) and optimum amounts of CoI and methylene blue, the Michaelis constant (K_m) was found by the method of Lineweaver & Burk (1934) to be 7×10^{-4} M.

Absorption spectrum. The spectrum of the purest enzyme preparations showed a peak at about 280 mμ., but no other bands of intense absorption. Since no band appeared in the vicinity of 340 mμ. on addition of sorbitol, it may be concluded that no CoI is bound to the dehydrogenase.

Substrate specificity. The enzyme is highly specific for D-sorbitol and L-iditol (see below). Many other compounds containing alcohol groups were tested, but glyceraldehyde was the only one oxidized by the most highly purified preparations of the enzyme. In the purest preparations DL-glyceraldehyde was oxidized at 12% of the rate (determined spectrophotometrically) found for D-sorbitol.

In manometric experiments enzyme (preparation *A*) supplied with methylene blue and CoI did not oxidize L-malate, pyruvate, DL-lactate, glucose or fructose. Examined spectrophotometrically, purified preparations (*A*) displayed no activity towards the following: D-mannitol, dulcitol, inositol, L-sorbose, D-fructose, D-glucose, D-gluconate, L-ascorbic acid; DL-lactate, pyruvate, dihydroxyacetone, DL-β-hydroxybutyrate; α- and β-glycerophosphate, glycerol, glycerate, glycollate; choline, ethanolamine, DL-serine, DL-threonine; glycolaldehyde diethylacetal, ethylene glycol, ethanol, *n*-propanol, *n*-butanol, and *n*-pentan-1-ol. Substrates were tested at concentration of 0·01 M, except the last six which were tested at 0·002 M.

Preparations of high activity possessed negligible aldolase, hexokinase or fructokinase activity, and only slight phosphatase activity when tested at pH 6·0 (succinate buffer) and 9·0 (veronal buffer) with β-glycerophosphate as substrate.

Inhibitors. Table 9 shows the effect of various inhibitors on sorbitol dehydrogenase. The action of boric acid may be due either to formation of a weakly dissociated complex with sorbitol, or to true inhibition of the enzyme. In spectrophotometric experiments it was found that 0·028 M-boric acid, pH 8·0, largely inhibited both the oxidation of sorbitol and the reduction of fructose and sorbose. Since sorbose has no pair of adjacent *cis*-hydroxyl groups, it is unlikely to form a complex with boric acid, and true inhibition of the enzyme is more probable in this case.

Table 9. *Effect of inhibitors on sorbitol dehydrogenase*

(Flasks contained 0·50 ml. enzyme (preparation *A*), 0·25 ml. sorbitol (0·016 M), 0·25 ml. CoI (12% purity, $1{\cdot}4 \times 10^{-4}$ M), 1·00 ml. potassium phosphate, pH 8·0 (0·063 M), inhibitor and water to give a final volume of 3·20 ml. Methylene blue (0·2 ml.; $9{\cdot}8 \times 10^{-4}$ M) was added from the side arm after equilibration. Gas, air. Time, 20 min. Temp. 38°.)

Inhibitor	O_2 uptake (μl.)	Inhibition (%)
Nil	215	—
Sodium borate, pH 7·8 (0·02 M)	10	95
Sodium iodoacetate (0·01 M)	148	31
Urethane (0·20 M)	206	4
NaN_3 (0·02 M)	230	−7
Mannitol (0·02 M)	203	5
Dulcitol (0·02 M)	208	3
Malonate (0·01 M)	210	2
n-Octanol (saturated solution)	198	8
KCN, pH 8 (0·01 M)	104	53
Na_2S, pH 8 (0·01 M)	228	−6

Distribution. Sorbitol dehydrogenase activity in other tissues of the rat and in the livers of various species was estimated by the increase in oxygen uptake of homogenates due to addition of sorbitol under the conditions shown in Table 10. In the first series of experiments the tissues were homogenized in 4 vol. of ice-cold 0·01 M-potassium phosphate, pH 8·0, adjusted to pH 5·0, centrifuged and the supernatant used for activity determination. The extract was kept at 0–2° until used. Since the loss of activity incurred in preparing this extract varied greatly in different tissues, the whole homogenate was used in a second series of experiments. With this procedure there is the disadvantage of more rapid loss of activity during both the equilibration and experimental periods. The two procedures gave results (Table 10) which differ significantly for rat kidney and for cat and rabbit liver, but which are otherwise parallel. Although only semi-quantitative, they show that sorbitol dehydrogenase is confined to liver and kidney in the rat, and is widely distributed in the livers of other species.

Reversibility of sorbitol oxidation

The time course of sorbitol oxidation was followed by measuring the rate of $CoIH_2$ formation when sorbitol and CoI reacted in the presence of a buffered sorbitol-dehydrogenase preparation. The reaction proceeded at exactly the same rate in the presence of phosphate as of glycylglycine buffer of similar pH and concentration.

When D-fructose was added to the reaction mixture after it had approached equilibrium, there was an immediate and progressive decline in $CoIH_2$ concentration until a second equilibrium state was reached. L-Sorbose, when added instead of fructose, also reoxidized $CoIH_2$ but at a slower rate (Fig. 5). D-Glucose had no effect. These experiments suggest

Table 10. *Distribution of sorbitol dehydrogenase activity*

(Flasks contained 0·50 or 1·00 ml. of whole homogenate or extract, 0·25 ml. sorbitol (0·017 M, replaced by water in controls), 1·00 ml. potassium phosphate, pH 8·0 (0·067 M), 0·25 ml. CoI (12% purity, 3×10^{-4} M), 0·20 ml. methylene blue (0·001 M) and water to give a final volume of 3·00 ml. With whole homogenate, flasks contained 0·30 ml. nicotinamide (0·01 M) and CoI was added from the side arm after equilibration; with extract, methylene blue was added from the side arm after equilibration. Inseals contained 0·20 ml. 2 N-NaOH. Gas, air. Temp. 38°.)

	O_2 uptake (μl./ml. homogenate or extract/initial 10 min.)					
	Extract			Homogenate		
Tissue	Sorbitol	Control	Difference	Sorbitol	Control	Difference
Rat liver	51	19	32	90	52	38
Rat kidney	10	8	2	60	24	36
Rat spleen	9	8	1	12	10	2
Rat heart	8	6	2	26	24	2
Rat lung	7	6	1	6	8	0
Rat skeletal muscle	8·5	6·5	2	6	8	0
Rat brain	7	5	2	0	2	0
Rat testis	12	7	5	4	0	4
Cat liver	13	17	—	103	44	59
Mouse liver	39	13	26	148	62	86
Guinea pig liver	47	16	31	115	41	74
Albino rabbit liver	9	10	0	105	37	68
Wild rabbit liver	31	20	11	140	70	70
Frog liver	11·5	7·5	4	55	40	15

that D-sorbitol is reversibly oxidized to L-sorbose and D-fructose in presence of sorbitol dehydrogenase and CoI. Since glucose was unable to reoxidize $CoIH_2$, it is presumably not a product of sorbitol oxidation. Glucose-6-phosphate and fructose-6-phosphate were also inactive with the enzyme when tested by this method, and neither glucose nor glucose-6-phosphate was converted to fructose or fructose-6-phosphate when incubated with the buffered enzyme and reduced CoI, either with or without adenosinetriphosphate and magnesium ions.

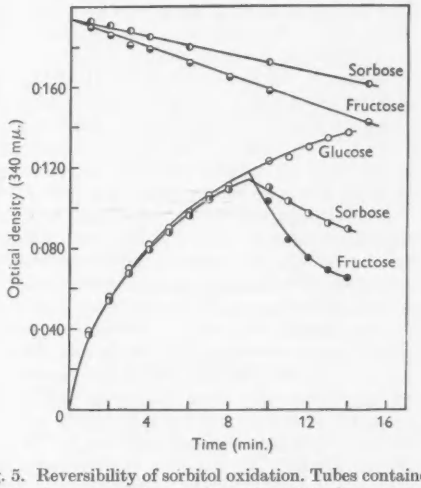


Fig. 5. Reversibility of sorbitol oxidation. Tubes contained 0·50 ml. enzyme (preparation *B*), 0·50 ml. potassium phosphate, pH 7·9 (0·033 M), substrate, CoI or $CoIH_2$ (3×10^{-5} M) and water to give a final volume of 3·00 ml. ◐—◐, $CoIH_2$ and L-sorbose (0·038 M). ◒—◒, $CoIH_2$ and D-fructose (0·038 M). ○—○, CoI and D-sorbitol (0·0033 M); after 9 min. 0·05 ml. D-glucose (0·0041 M) added. ●—● CoI and D-sorbitol (0·0033 M); after 9 min. 0·05 ml. D-fructose (0·0041 M) added. ◑—◑, CoI and D-sorbitol (0·0033 M); after 9 min. 0·05 ml. L-sorbose (0·0041 M) added.

The initial rate of enzymic oxidation of $CoIH_2$ (used as sodium salt, 20% purity) by L-sorbose was 69% of the rate obtained with D-fructose (Fig. 5). The ketoses did not react with $CoIH_2$ in absence of enzyme.

Products of the oxidation of sorbitol by the dehydrogenase

The above experiments suggest that D-fructose and L-sorbose, but not D-glucose, are formed during the enzymic oxidation of D-sorbitol. The question of glucose formation was investigated by oxidizing sorbitol enzymically under aerobic conditions in the presence of CoI and methylene blue, and testing the deproteinized solution, freed of methylene blue, for glucose with glucose oxidase. The results showed the absence of glucose. The solution, however, gave a strong test for ketose and it was possible to demonstrate (Table 11) that the ratios, oxygen uptake : ketose formed (determined colorimetrically and calculated as fructose) : sorbitol utilized, were in accord, within the limits of experimental error, with the stoicheiometric requirements of the equation

$$C_6H_{14}O_6 + \tfrac{1}{2}O_2 \rightarrow C_6H_{12}O_6 + H_2O.$$

Because of the difficulty of estimating sorbitol by periodate in the presence of large amounts of hexose, incubation was continued in Exps. 1–6

Table 11. *Oxygen uptake, ketose formation and sorbitol disappearance*

(Flasks 1–6 contained 1·25 ml. enzyme (preparation *B*), flasks 7–11 contained 1·00 ml. In addition flasks contained 0·25 ml. Co I (12% purity, 2×10^{-4} M), 0·50 ml. phosphate, pH 8·0 (0·033 M), sorbitol and water to give a final vol. of 3·20 ml. Flasks 7–11 also contained 0·50 ml. of a diaphorase preparation. Methylene blue ($0·2$ ml.; $9·4 \times 10^{-4}$ M) added from side arm after equilibration. Exps. 1–6 continued till O_2 uptake ceased. Exps. 7–11, time, 2·25 hr. O_2 uptake corrected for that in absence of substrate (16 μl./hr.). Gas, air. Temp. 38°.)

Exp. no.	Initial sorbitol concn. ($\times 10^{-6}$ M)	Sorbitol disappearing (μmol.)	O_2 uptake (μatom)	Ketose formed (calc. as fructose) (μmol.)
1	0·61	2·0	2·0	—
2	1·56	5·0	5·4	—
3	3·13	10·0	10·1	—
4	4·54	15·0	15·4	—
5	6·05	20·0	19·2	—
6	9·07	30·0	27·2	—
7	1·26	—	4·7	4·2
8	2·52	—	7·0	7·1
9	3·16	—	7·9	8·2
10	3·79	—	9·6	9·1
11	4·38	—	9·7	10·2

(Table 11) until oxygen uptake ceased. On the assumption that all the sorbitol had been oxidized, the amount disappearing was taken as equal to the amount initially added. The agreement obtained (Table 11) with the theoretical values justifies the assumption made for the purpose of calculating ketose concentration, that the ketose was indeed fructose. Under the conditions of the experiment no carbon dioxide is formed during oxidation. In larger-scale experiments (Table 12) it was confirmed that fructose was the chief product of the enzymic oxidation of sorbitol.

Table 12. *Fructose formation from sorbitol*

(Enzyme (93 ml., preparation *B*) incubated with sorbitol (0·048 M) and Co I (12% purity, $9·6 \times 10^{-5}$ M), dissolved in 5·0 ml. potassium phosphate, pH 7·9 ($9·6 \times 10^{-3}$ M) and 6·0 ml. methylene blue ($9·5 \times 10^{-4}$ M). Mixture (104 ml.) divided among six 150 ml. Erlenmeyer flasks. Time, 14 hr. Gas, O_2. Temp. 38°. Optical rotation measured and analyses performed on deproteinized filtrate, freed of methylene blue. Controls without substrate had zero rotation, and contained no reducing sugar or ketose.)

	Concn. (mg./ml.)
Total reducing sugar	3·02
Fructose (calc. from $\alpha_D = 1·06 \pm 0·01°$ in water; *l*, 4)	2·87
Ketose (calc. as fructose from colorimetric determination)	2·66

The formation of fructose was demonstrated by the preparation of osazones. A solution of reaction products prepared by the method outlined in Table 13 (4 ml.) gave characteristic crystals of phenylglucosazone. In another experiment the entire deproteinized solution, freed of methylene blue, was evaporated to dryness under reduced pressure (bath temperature 40°), the residue extracted with boiling 95% ethanol, and the extract filtered and evaporated to dryness under reduced pressure. The residue was taken up in 1·5 ml. of α-methylphenylhydrazine sulphate reagent (Neuberg & Mandl, 1946) and incubated at 25° for 1 hr. As controls 200 mg. of D-glucose and 200 mg. of D-fructose were treated separately with the reagent in the same way. Crystallization of α-methylphenylfructosazone could not be induced within this period at 25° as reported by Neuberg & Mandl (1946), and in other experiments only crude crystalline material was obtained after several days at 2°. After incubation at 25° the osazone was therefore extracted according to Bacon & Bell (1948), and recrystallized successively from chloroform-light petroleum, 20% (v/v) ethanol, ethyl acetate and finally ethyl acetate-light petroleum (b.p. 40–60°). The last mixture proved to be the best solvent for crystallization, readily giving fine yellow to orange needles, free from contaminating amorphous material which was otherwise difficult to remove. Observed microscopically on a melting-point block the crystals melted at 151–153° (decomp.), while the sample prepared simultaneously from authentic D-fructose had m.p. 152·5–153·5° (decomp.). In capillary tubes heated in a bath these samples had m.p.'s 130–132° (decomp.) and 133–135° (decomp.) respectively, and gave a mixed m.p. 134–137° (decomp.). Further recrystallization did not raise the melting point. Glucose gave no crystalline product.

While this establishes fructose as the main product of the enzymic oxidation of sorbitol there remains the possibility that a smaller quantity of L-sorbose is formed. The enzymic reduction of sorbose by Co I H_2 seems to indicate that this may be the case. Sorbose, however, could not be detected in experiments in which the accumulated products of aerobic sorbitol oxidation were fermented with baker's yeast and subsequently tested for ketose.

Table 13 *Enzymic reduction of sorbose and fructose*

(Enzyme (2·00 ml., preparation *B*) incubated with 0·50 ml. hexose (0·02 M), 0·20 ml. Co I H_2 (20% purity, 1×10^{-4} M) and 0·30 ml. potassium phosphate, pH 7·9 (0·02 M). Time, 3 hr. Temp. 38°. Deproteinized with $Ba(OH)_2$-$ZnSO_4$, and 3·00 ml. of filtrate used for sorbose determination after fermenting with 0·5 vol. of a 10% (w/v) suspension of washed baker's yeast in potassium phosphate, pH 6·0 (0·20 M) for 6 hr. at 25°.)

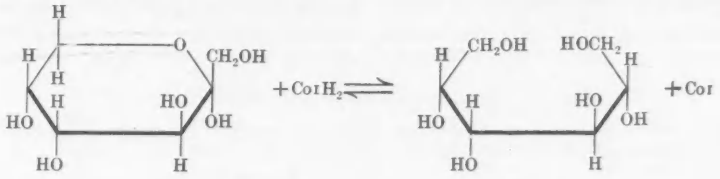
	Initial substrate (0·02 M)	After incubation: Fermentable ketose (μg.)	After incubation: Unfermented ketose (μg.)
Complete system	Fructose	640	48
	Sorbose	0	840
Co I H_2 omitted	Fructose	806	37
	Sorbose	0	1043
Heat-inactivated enzyme	Fructose	902	42
	Sorbose	0	992

An attempt to accumulate sorbose more rapidly was made by incubating sorbitol with enzyme and Co I in the presence of fructose. Under these conditions conversion of fructose to sorbitol should maintain a steady rate of sorbose formation by keeping the concentration of sorbitol and Co I constant. No sorbose could be detected in the deproteinized solution. The sensitivity of the method is such as to permit the conclusion that if sorbose is formed by enzymic oxidation of sorbitol it constitutes less than 1% of the total ketose produced.

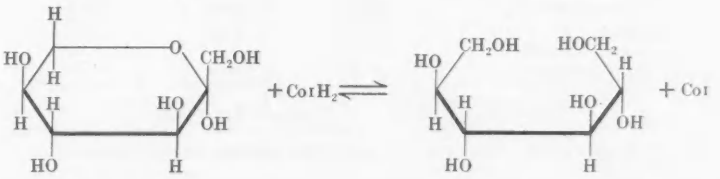
An attempt to demonstrate the interconversion of L-sorbose and D-fructose through D-sorbitol as an intermediate was made by incubating each of the hexoses with buffered sorbitol dehydrogenase and produced from sorbose. The small amount of unfermented 'ketose-reacting' substance present both in controls and in experiments with fructose was shown to be derived from the yeast.

Oxidation and antiketogenic effect of L-*iditol*

The absence of detectable quantities of sorbose amongst the products of sorbitol oxidation, in spite of the reduction of L-sorbose by Co I H_2 in the presence of the dehydrogenase, suggests that sorbose may be reduced to some other hexitol. When the pyranose structures of D-fructose and L-sorbose are compared it may be seen that the reversible reduction of D-fructose to D-sorbitol corresponds stereochemically to the reversible reduction of L-sorbose to L-iditol.



α-D-Fructopyranose D-Sorbitol



L-Sorbopyranose L-Iditol

Co I H_2 (Table 13). Although an appreciable disappearance of each hexose occurred, no increase in unfermentable ketose above that in the control with heat-inactivated enzyme was detected in the presence of fructose, and no fermentable sugar was

It would have been impossible to test this hypothesis without the kindly assistance of Prof. G. Bertrand, who generously presented crystalline specimens of D- and L-iditol (synthetic) and L-iditol (natural 'sorbierite'). It was found that L-iditol

(natural and synthetic) was readily oxidized by rat-liver slices (Table 14) and by Co I in presence of the dehydrogenase preparation (Fig. 6). On the other hand, D-iditol was not oxidized under the same conditions, and the enzyme apparently has little affinity for this compound since D-iditol, like D-mannitol and dulcitol, does not competitively inhibit the oxidation of D-sorbitol or L-iditol by the dehydrogenase.

Table 14. *Oxidation and antiketogenic effect of* L-*iditol*

(Liver slices from a rat fasted 24 hr. were incubated in conical Warburg vessels containing 3·00 ml. of phosphate saline, pH 7·4. Inseals contained 0·20 ml. 2N-NaOH. Time, 2 hr. Gas, O_2. Temp. 38°.)

Substrate (0·01 M)	$-Q_{O_2}$	Q_{Keto}
Nil	10·7	2·78
Sorbitol	14·3	0·97
L-Iditol ('sorbierite')	12·8	0·88
L-Iditol (synthetic)	11·9	0·82
D-Iditol (synthetic)	11·1	2·94

It was of considerable interest to determine whether L-iditol, being readily oxidized by rat liver, could depress the spontaneous ketogenesis of liver slices from starved rats, or whether the antiketogenic effect was specific to sorbitol. It was found that L-iditol is effectively antiketogenic to the same extent as sorbitol, whilst D-iditol, like mannitol and dulcitol, has no effect (Table 14).

Products of iditol oxidation

The oxidation of L-iditol by the coenzyme-dehydrogenase system produced a non-fermenting ketose. When the concentration of ketose was calculated on the assumption that the colour developed was due to sorbose, agreement was obtained between the amounts of $Co\,I\,H_2$ and sorbose formed in the oxidation of L-iditol (Table 15). Although the ketose has not been isolated, it is almost certainly L-sorbose.

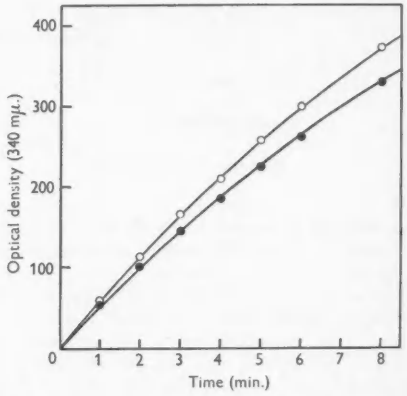


Fig. 6. Oxidation of L-iditol. Tubes contained 0·50 ml. enzyme (preparation *A*), 2·15 ml. potassium phosphate, pH 7·8 (0·0073 M) and 0·20 ml. Co I (60% purity, 0·0075 M). Hexitol (0·01 ml.; 0·0063 M) added at zero time. ○—○, D-Sorbitol; ●—●, L-iditol.

The oxidation of L-iditol by rat-liver slices produced glucose and a non-fermenting ketose, probably sorbose (Table 16). Compared with the products of sorbitol oxidation by slices, much less glucose and much more ketose accumulated.

Table 15. *Products of the reaction of* L-*iditol with* Co I

(Tubes contained 0·50 ml. dehydrogenase (preparation *B*), 2·15 ml. potassium phosphate, pH 7·8 (0·0073 M), 0·20 ml. Co I (60% purity, 0·0075 M) and 0·10 ml. hexitol (0·0063 M). Temp. 20°. Time, about 3 hr.)

	Reaction products		
Hexitol added	$Co\,I\,H_2$ (μmol.)	Total ketose (μmol.)	Unfermentable ketose (μmol.)
Sorbitol	0·74	0·73*	0·00
L-Iditol ('sorbierite')	0·76	0·85†	0·79†
L-Iditol (synthetic)	0·74	0·80†	0·79†
D-Iditol (synthetic)	0·00	0·00	—

* Calculated as fructose. † Calculated as sorbose.

Table 16. *Products of the oxidation of* L-*iditol by rat-liver slices*

(Slices from a rat fasted 24 hr., suspended in 12·0 ml. of phosphate saline, pH 7·4, in 150 ml. Erlenmeyer flasks. Iditol concentration 0·01 M. Time, 4 hr. Gas, O_2. Temp. 38°. Suspension fluid deproteinized with $Ba(OH)_2$-$ZnSO_4$. For determination of reducing sugar, glucose and ketose see Analytical methods section.)

Dry wt. of slices (mg.)	Iditol added (mg.)	Reducing sugar (calc. as glucose) (mg.)	Glucose (mg.)	Ketose (calc. as sorbose) (mg.)
103·3	Nil	1·0	0·6	0·0
98·6	21·6	6·8	5·7	2·7

Equilibrium constant for sorbitol-fructose reaction

The equilibrium constant for the reversible oxidation of sorbitol to D-fructose can be calculated from the concentrations of sorbitol, fructose, CoI and $CoIH_2$ at equilibrium. By making the concentrations of sorbitol and fructose high compared with those of coenzyme, the former can be regarded as remaining at their initial levels. Hence the equilibrium constant can be obtained from the values of the initial sorbitol and fructose concentrations and the determined CoI and $CoIH_2$ concentrations. $CoIH_2$ was estimated by the spectrophotometric method, and CoI was calculated by difference from total assayed coenzyme concentration.

In a preliminary experiment the total coenzyme concentration was calculated from the dilution of a stock coenzyme solution, assayed by the dithionite method (LePage, 1947). From a series of eight reaction mixtures with varying ratios of sorbitol to fructose, the mean value of the equilibrium constant was found to be 0·230. In the second experiment (Table 17) the total coenzyme concentration was determined by enzymic reduction with 0·118M-sorbitol, since at equilibrium the $CoIH_2$ may be equated to the total coenzyme present without appreciable error.

Table 17. *Equilibrium constant for reversible oxidation of sorbitol*

(Tubes contained 0·59 ml. enzyme (preparation *B*), 0·49 ml. potassium phosphate, pH 8·0 (0·03M), 0·32 ml. CoI (12% purity, $1·2 \times 10^{-4}$M), sorbitol, fructose and water to give a final vol. of 3·40 ml. Temp. 20·0±0·5°. Time, approx. 1·5 hr., until $E_{340\,m\mu}$ was constant.)

Concentrations of reactants				
Sorbitol ($\times 10^{-2}$M)	Fructose ($\times 10^{-2}$M)	CoI ($\times 10^{-5}$M)	$CoIH_2$ ($\times 10^{-5}$M)	K
10·59	0·72	2·64	9·65	0·249
10·00	1·09	3·79	8·50	0·244
9·41	1·45	4·62	7·67	0·256
8·82	1·81	5·50	6·79	0·253
8·23	2·17	6·52	5·77	0·233
7·65	2·52	7·03	5·26	0·246
6·47	3·26	8·43	3·86	0·231
5·29	3·98	9·48	2·81	0·223
4·12	4·70	10·19	2·10	0·235
2·94	5·43	10·96	1·33	0·224

The data for the second determination shown in Table 18 give $K(20°) = 0·240$ with a 95% confidence interval of ±0·013. This corresponds to $\Delta F° = 832$ cal. in the oxidation of sorbitol by CoI. Taking E_0 at 30° = −0·072 V. and

$$\Delta E / \Delta T = -0·00043 \text{ V.}$$

for $CoI/CoIH_2$ (Borsook, 1940), E_0 for the system sorbitol/fructose at 20° is $+0·185 - (0·058 \times pH)$ V. = −0·283 V. at pH 8·0.

This value for E_0' at pH 8 is in good agreement with the behaviour of the system with oxidation-reduction indicators (Table 8). From the above data $\Delta F°$ at 20° for the reaction

D-Sorbitol (aq.) → D-fructose (aq.) + $2H^+ + 2e$

is $8·5 \times 10^3$ cal./mol. at pH 8·0.

DISCUSSION

The demonstration of the existence of sorbitol dehydrogenase reacting with CoI provides an explanation for the oxidation of sorbitol by perfused dog liver (Embden & Griesbach, 1914), cat-liver brei (Breusch, 1942, 1943) and rat-liver slices and homogenates. The presence of dehydrogenase activity in liver homogenates from all mammalian species examined suggests that the enzyme has widespread occurrence.

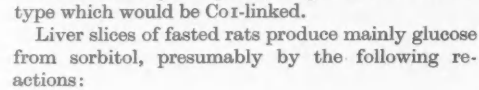
It has been established with partially purified dehydrogenase preparations from rat liver that the product of sorbitol oxidation is D-fructose, which is probably metabolized further in homogenates and intact cells via the glycolytic sequence of reactions. It has been shown also that the dehydrogenase oxidizes L-iditol, the evidence indicating that the product is L-sorbose. In the presence of buffered dehydrogenase preparations and coenzyme the oxidation both of L-iditol and of sorbitol is reversible (Figs. 5 and 6), and the equilibrium constant for the latter reaction has been determined (Table 17). It is reasonable to assume that the equilibrium constant for the iditol-sorbose reaction has a similar value.

The dehydrogenase is highly specific, D-sorbitol and L-iditol being the only substrates reacting significantly. The slow oxidation of glyceraldehyde by liver extracts is probably due to another component of the enzyme preparation since the rate of oxidation of this substrate was very low in the purest preparations of the enzyme. Since D-glucose and D-gluconate are not oxidized, the enzyme requires the presence of a primary alcohol group at C-1. Furthermore, the inability of the enzyme to oxidize D-

mannitol and dulcitol indicates that the configuration of the substrate at C-2 and C-4 must conform to the pattern in D-sorbitol for reaction to occur. No direct evidence has been obtained regarding the configuration at C-3, the only substrate tested which differed from D-sorbitol in its configuration at this centre (D-iditol) differing also in the configuration at C-2 and C-4.

In the above discussion it has been assumed that a single enzyme is responsible for the sorbitol-fructose and iditol-sorbose reactions, but it is possible that different dehydrogenases are involved. If the enzymes are not identical it is likely that the reactions are catalysed by dehydrogenases of the same type which would be CoI-linked.

Liver slices of fasted rats produce mainly glucose from sorbitol, presumably by the following reactions:



The extent to which a glucose-sparing effect of sorbitol contributes to the accumulation of glucose could not be assessed with the techniques employed. Only small amounts of fructose can be detected in the suspension medium (Table 3). Assuming that the accumulated glucose is derived directly from sorbitol, it may be concluded that in liver slices of fasted rats oxidation of sorbitol to fructose and conversion of fructose to glucose proceed much more rapidly than hexose oxidation.

The physico-chemical characteristics of the enzymic oxidation of sorbitol afford no obvious explanation of the marked antiketogenic action (Tables 1 and 14). The substrate-enzyme affinity ($K_m = 7 \times 10^{-4}$ M), the equilibrium constant (K, 0·240) and the corresponding redox potential (E'_0 at pH 8·0 = −0·283 V.) are of the same order as those of several other dehydrogenases. However, the fact that both D-sorbitol and L-iditol are antiketogenic despite the formation of different oxidation products, suggests that the dehydrogenation itself is the source of the antiketogenesis. Two other effectively antiketogenic compounds, glycerol and glyceraldehyde, may well owe their effect also to rapid dehydrogenation. The actual mechanism by which the dehydrogenation interacts with fatty acid oxidation must at present remain a matter for speculation.

SUMMARY

1. The oxidation of D-sorbitol in rat-liver slices and homogenates is catalysed by a dehydrogenase using coenzyme I. The dehydrogenase also oxidizes L-iditol; the specificity is discussed.

2. The product of the oxidation of D-sorbitol by the partially purified dehydrogenase is D-fructose. L-Iditol is probably oxidized to L-sorbose.

3. The main product of the oxidation of sorbitol by liver slices is glucose. Very small quantities of fructose are also formed. Oxidation of L-iditol by slices produces small amounts of glucose and sorbose.

4. Sorbitol and iditol considerably lower the spontaneous ketogenesis of liver slices from fasted rats.

5. The oxidation of sorbitol by coenzyme I to fructose is reversible; the equilibrium constant is 0·240 ± 0·013. The oxidation of L-iditol to L-sorbose is also reversible.

6. The optimum conditions for the oxidation of sorbitol by the liver dehydrogenase are pH 7·9–8·1 and 38–40°. $K_m = 7 \times 10^{-4}$ M.

7. Sorbitol dehydrogenase is found in the rat only in liver and kidney. It appears to be widely distributed in mammalian liver.

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The Action of 2:4-Dinitrophenol on Oxidative Phosphorylation

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In a previous paper (Judah & Williams-Ashman, 1951) the effect of a number of compounds was tested on oxidative phosphorylation in cell-free systems. 2:4-Dinitrophenol (DNP) was selected for further study because of widespread interest in this compound and because its action was particularly clear-cut and reproducible.

The starting point of the present work was the investigation of the inhibition of pyruvate oxidation by DNP; this has led to an attempt at localization of the action of DNP and to a study of the 'replacement' of inorganic orthophosphate by DNP (Loomis & Lipmann, 1948) in systems deficient in phosphate. Some experiments on the action of azide and observations on the effect of thyroxine on oxidative phosphorylation are also included.

METHODS

All methods are as described previously (Judah & Williams-Ashman, 1951) with the following exceptions.

Enzyme preparations. Preparations of mitochondria from liver and kidney of rat and rabbit were invariably carried out by the method of Schneider (1948), using 0·25 M-sucrose as the medium. This method gives a uniform preparation free of cell debris, erythrocytes and cell-nuclei, which form a large part of such preparations as 'cyclophorase' (Green, Loomis & Auerbach, 1948). For homogenization, the apparatus of Potter & Elvehjem (1936) was used. A blender yielded suspensions which were far less active and which invariably failed to show proportionality between tissue concentration and respiratory rate. Furthermore, the yields of mitochondria were low when the tissue was disintegrated in the blender. All preparations were carried out at 0°; the mitochondrial suspensions obtained were found to be stable for several hours when kept cold; preparations stored for as long as 6 hr. showed no decline in the efficiency of oxidative phosphorylation. Considerable uniformity in properties was observed from one preparation to another (O_2 uptake with pyruvate being approx. 500 μl. O_2/mg. N/hr.), and no inactive preparations have been encountered in a series of several hundred.

The mitochondria tolerated repeated resuspension and sedimentation without appreciable loss of activity, particular preparations having been subjected to a cycle of five or six such operations. In washing the particles care was taken to ensure an even suspension in the sucrose medium. This was achieved by the use of a mechanically rotated pestle which fitted the centrifuge tube loosely. A few seconds sufficed to disperse the tightly packed particles, which were dispersed in six to eight 50 ml. cellulose tubes, washings being conducted with 25–30 ml. 0·25 M-sucrose per tube.

Microsomes (submicroscopic particles) were prepared as described by Schneider (1948), a force of 25,000 *g* being applied for 1 hr. to the suspension in 0·25 M-sucrose.

Reagents. Crystalline sodium or potassium pyruvate were prepared from commercial pyruvic acid after redistillation *in vacuo*. α-Ketoglutaric acid was obtained from Nutritional Biochemicals, Inc., Cleveland, Ohio. Inorganic pyrophosphate was an analytical reagent; on chromatography in ethyl acetate-pyridine (Hanes & Isherwood, 1949), it showed only a trace of inorganic ortho-P and otherwise gave a single homogeneous spot.

Estimations. Orthophosphate was estimated by Ba and Mg precipitation (Umbreit, Burris & Stauffer, 1949) and by Ca precipitation at neutral pH by an unpublished method of the writer. Phosphate balances were determined as described by LePage in Umbreit *et al.* (1949).

Nucleic acid estimations were carried out by the method of Schmidt & Thannhauser (1945).

Pyruvate and α-ketoglutarate were determined by the method of Friedemann & Haugen (1943), using either a Beckman Model DU spectrophotometer or a Unicam diffraction-grating instrument.

Ferricyanide was determined spectrophotometrically by its absorption at 420 mμ. after deproteinization with trichloroacetic acid.

N was determined by the micro-Kjeldahl method. Steam volatile acid was estimated by the method of Long (1938).

RESULTS

The oxidation of pyruvate and its inhibition by DNP. Preliminary experiments showed that the following medium gave satisfactory results. Inorganic orthophosphate, 0·003–0·03 M; adenosine-5-phosphate, 0·001 M; magnesium sulphate, 0·0067 M; potassium chloride, 0·025 M; cytochrome *c*, 0·00001 M; pyruvate, 0·01 M; fumarate, malate or succinate, 0·0003–0·001 M; 0·5 ml. mitochondrial suspension in 0·25 M-sucrose and water to a final volume of 3·0 ml. This medium is very similar to that described by Kennedy & Lehninger (1949), and by Green *et al.* (1948).

Green *et al.* (1948) and indicating that the enzyme catalysing the reversible decarboxylation of oxaloacetic acid is present in the mitochondria. It is clear that in the presence of the enzyme, pyruvate and carbon dioxide, oxaloacetate in amounts sufficient to initiate the condensation to citrate will be formed (Utter & Wood, 1946). Determination of steam-volatile acid and of pyruvate disappearing show that a one-step oxidative decarboxylation does not occur in the absence of added primer. Several attempts were made to obtain this reaction in mitochondrial systems. None was successful. The addition of aneurin pyrophosphate, coenzyme I (Co I) and

Table 1. *Complete oxidation of pyruvate*

(The systems were as described in the text, except that pyruvate additions are as shown in the table. All values shown are corrected for blanks. Temp. 38°. Gas phase: air. The primer was fumarate in every case: 1 μg.mol. in Exp. 1, 2 μg.mol. in Exp. 2, and 3 μg.mol. in Exps. 3 and 4.)

Exp. no.	Source of mitochondria	Pyruvate added (μg.mol.)	Pyruvate at end of experiment (μg.mol.)	O_2 uptake (μg.atoms) Observed (blank subtracted)	Expected	Blanks	Time on bath (min.)
1	Rat kidney	4·0	0·0	18·0	20·0	7·0	180
2	Rat liver	4·0	0·0	19·1	20·0	14·6	165
3	Rat kidney	4·0	0·1	19·0	19·5	20·9	120
4	Rabbit kidney	10·0	0·0	48·5	50·0	17·8	110

Initially the potassium salts of all reagents were used, but it was found that sodium salts would serve equally well in the presence of 0·025 M-potassium chloride. In all the experiments cited below, sodium salts were used.

Table 1 shows the complete oxidation of pyruvate by several mitochondrial preparations. The observed oxygen uptakes agree reasonably well with theory; the complete removal of pyruvate was checked by chemical estimations on the flask contents. For these experiments pyruvate was placed in the side arms of the flasks, and to avoid temperature inactivation of the enzyme in the absence of substrate, a dicarboxylic acid (malate, fumarate or succinate in the concentrations given above) was placed in the main compartment of the flask. Addition of catalytic amounts of these acids (primers) is required to initiate or prime the oxidation of pyruvate by providing oxaloacetate necessary for the condensation of pyruvate (or a degradation product of pyruvate) to form citrate. This permits its complete oxidation via the tricarboxylic acid cycle (Krebs & Johnson, 1937; Green *et al.* 1948). Blanks were run simultaneously without added pyruvate, and their oxygen consumption subtracted from that of the experimental flasks.

In Table 2 will be seen the effect of leaving out primer, adenylic acid and cytochrome *c*; loss of cytochrome *c* does not result in a great decrease of respiratory rate. The primer in this experiment was sodium bicarbonate, confirming the observation of adenosinetriphosphate (ATP) failed to influence this result. Water suspensions of mitochondria were also tested, but these failed to show any activity whatever.

Table 2. CO_2 *priming of pyruvate oxidation; the effect of leaving out primer, adenosine-5-phosphate and cytochrome* c

(The complete system was as described in the text except that 30 μg.mol. of $NaHCO_3$ were added to prime the reaction; the system contained Na_2HPO_4, pH 7·40, 0·03 M, as buffer. Rabbit-kidney mitochondria. Temp. 38°. Gas phase: air.)

System	O_2 uptake (μl./30 min.)	Steam volatile acid as μg.mol. acetate
Complete	214	0·45
Adenosine-5-phosphate	57	0·90
No primer	15	—
No cytochrome *c*	180	—

Schneider & Potter (1949) have studied the oxaloacetic oxidase of rat-liver mitochondria and have claimed that the addition of the microsomes to the mitochondria gave rise to increased respiratory rates, though the microsomes were devoid of oxidase activity. These experiments were repeated to determine whether the extra oxygen uptake was associated with an increased phosphate uptake. It was found that the microsomes had a negligible action on oxygen uptake and P/O ratio, both in the presence and absence of fluoride. However, these

Table 3. *Inhibition of pyruvate oxidation by DNP*

(The systems were as described in the text. The analyses for pyruvate and acetate were carried out at the end of the experiments, when no further movement of the manometers could be observed. Exps. 1 and 2, the flask contained 10 μg.mol. pyruvate; Exp. 3, 22 μg.mol. pyruvate.)

Exp. no.	Inhibitor	Primer	O_2 uptake (μl.) 1st 20 min.	O_2 uptake (μl.) 2nd 20 min.	O_2 uptake (μl.) Total	Pyruvate disappearing (μg.mol.)	Steam volatile acid as μg.mol. acetate	Time (min.)
1	Nil	0·001 M-Fumarate	342	210	650	10·0	0·0	100
	5×10^{-5} M-DNP	0·001 M-Fumarate	300	90	480	9·7	2·2	100
2	Nil	0·01 M-$NaHCO_3$	270	190	550	10·0	0·0	100
	5×10^{-5} M-DNP	0·01 M-$NaHCO_3$	85	60	202	7·7	4·0	100
3	Nil	0·01 M-$NaHCO_3$	224	250	1000	21·0	0·0	140
	10^{-4} M-DNP	0·01 M-$NaHCO_3$	82	37	170	12·5	5·5	140

Table 4. *Reversal of DNP inhibition of pyruvate oxidation by ATP and* Co I

(The control system was as described in the text, 30 μg.mol. of pyruvate and 1 μg.mol. of succinate being substrates. The additions were made from side arms at 30 min. from the start of the experiment. ATP 160 μg. of labile P; Co I 2·5 μg.mol. Rat-liver mitochondria. Temp. 38°. Gas phase: air.)

System	Addition	O_2 uptake (μl. O_2 at 10 min. intervals after addition) 1st 10 min.	2nd 10 min.	3rd 10 min.	4th 10 min.
Control	ATP + Co I	138	139	139	135
Control	Nil	100	94	92	90
10^{-4} M-DNP	Nil	28	21	10	5
10^{-4} M-DNP	ATP + Co I	108	111	88	48
10^{-4} M-DNP	Co I	65	74	57	44

experiments differed from those of Schneider & Potter in that the bath temperatures employed were 15°, while the latter investigators worked at 38°. In the presence of sodium fluoride and at 15°, an uncorrected P/O ratio of 3·4 was obtained for pyruvate oxidation.

DNP at low concentrations (approx. 10^{-4} M) will inhibit the oxidation of pyruvate in these systems. The inhibition is variable. In some preparations immediate depression of the oxygen uptake is observed; in others the inhibition becomes apparent after some 20–30 min. incubation, during which time the rate of respiration in the DNP-poisoned systems is the same as, or slightly higher than, that in the controls. Typical examples of such experiments are shown in Table 3. No α-ketoglutarate accumulated in the flasks.

Reversal of DNP effect. Experiments were then undertaken to reverse this inhibitory effect of DNP, and it was found that reversal could be obtained by the addition of ATP or of Co I from the side arms of the flasks at the time when inhibition became apparent. The addition of aneurin pyrophosphate or of aneurin hydrochloride together or separately did not have this effect; nor did they influence the effect of ATP and Co I. Table 4 shows an experiment in which ATP (2·6 μg.mol.) and Co I (2·5 μg.mol.) were added. The figures show the oxygen uptakes from the moment when the inhibition became apparent and the additions were made. There was a considerable increase in the respiratory rate for some 20 min. after the additions.

Lipmann & Kaplan (1949) have claimed that the DNP inhibition of pyruvate oxidation can be reversed by the addition of dicarboxylic acids of the citric acid cycle. This claim has been confirmed, and in Table 5 are given interval oxygen uptakes for a system oxidizing pyruvate in the presence of 1·0 and 3·0 μg.mol. of L-malate, with and without DNP.

Table 5. *Reversal of DNP inhibition of pyruvate oxidation by extra malate and ATP*

(All systems contained 30 μg.mol. of pyruvate and 1 μg.mol. of malate. Extra malate added as shown, ATP when added was equivalent to 390 μg. of labile P.)

System	Addition	O_2 uptake (μl.) 2nd 15 min.	4th 15 min.
Control	ATP	295	250
10^{-4} M-DNP	ATP	232	183
Control	2 μg.mol. malate	296	270
10^{-4} M-DNP	2 μg.mol. malate	277	191
10^{-4} M-DNP	Nil	259	59

The extra malate had the effect of postponing the inhibitory effect of DNP, despite the fact that it was in the main compartment of the flask from the start of the experiment. In the same experiment ATP (6·3 μg.mol.) was added to an equivalent number of flasks, and it will be seen that its effect on the

inhibition was identical with that of malate, despite the fact that the adenosinetriphosphatase (ATP-ase) of the preparation would have destroyed the ATP long before its action became apparent.

It should be pointed out that the inhibition of pyruvate oxidation is fundamentally different from the inhibition of octanoate oxidation by DNP, and in keeping with this is the fact that ATP will not reverse the DNP effect on fatty acid oxidation.

To afford a single explanation for the effects of added ATP and extra malate on the inhibition of pyruvate oxidation, it must be supposed that the oxidation of malate via the tricarboxylic acid cycle gives rise to high-energy phosphate bonds even in the presence of DNP, which has been shown to inhibit, apparently completely, the phosphate uptake associated with the oxidations catalysed by the particles (Loomis & Lipmann, 1948; Cross, Covo, Taggart & Green, 1949; Judah & Williams-Ashman, 1951). This point was, therefore, further investigated, and experiments were undertaken to determine the possible site of DNP action.

Attempts to localize DNP action. A simple explanation of the problem would be to assume that DNP inhibited the net uptake of phosphate, permitting the formation of ATP which, however, was rapidly used up in side reactions before transphosphorylation to hexose could occur. One such side reaction is the breakdown of ATP by adenosinetriphosphatase (ATP-ase), but others might exist. Hunter & Hixon (1949*a*) have shown that by working at 15° very high P/O ratios can be obtained. The action of DNP on oxidative phosphorylation at this temperature was therefore tested in the hope that under optimal conditions any P uptake would become apparent. Table 6 shows an experiment with glutamate as substrate, in which no P uptake was apparent in the presence of DNP, though a P/O ratio as high as 3·8 was obtained in the controls.

Table 6. *Action of DNP on* P/O *ratio*

(The control system contained 30 μg.mol. L-glutamate as substrate. Inorganic orthophosphate, 0·02 M; adenosine-5-phosphate, 0·001 M; $MgSO_4$, 0·0067 M; NaF, 0·013 M; cytochrome *c*, 0·00001 M; KCl, 0·025 M; hexokinase, 0·2 ml.; fructose, 0·033 M; 0·5 ml. mitochondrial suspension in 0·25 M-sucrose and water to 3·0 ml. Time in bath 10 min. Temp. 15°. Gas phase: air.)

System	O_2 uptake (μg.atoms)	P uptake from inorganic orthophosphate (μg.atoms)	P/O ratio
Control	6·8	26·0	3·8
10^{-4} M-DNP	6·55	0·0	—

An attempt was therefore made to localize the steps at which phosphate uptake might occur and to determine the effect of DNP upon them.

Phosphorylation coupled to cytochrome c *oxidation; effect of DNP.* In the presence of rat-liver mitochondria, the addition of cytochrome *c* (0·0001 M) and L-ascorbic acid (0·01 M) resulted in a rapid oxygen uptake. Table 7 shows that a clear-cut phosphorylation takes place during this oxidation, the P/O ratio approaching a value of 1·0. In each experiment blanks were set up, leaving out cytochrome *c*, L-ascorbic acid or both, and the oxygen consumption and phosphate uptake in these flasks has been subtracted from the values observed in the complete systems. DNP abolishes the P uptake while causing a slight acceleration of oxygen uptake.

Because Lehninger (1949) has suggested that dehydroascorbic acid might be further oxidized by rat-liver particles, and thus account for a phosphate uptake, the disappearance of ascorbic acid was investigated. Flasks were set up with all components; without cytochrome *c*; without particles, and without particles or cytochrome *c*. Initial values were obtained from identical flasks which were deproteinized at the end of equilibration. The results of the experiments are shown in Table 8. Very good agreement was obtained between the oxygen uptakes and chemical estimations of ascorbic acid, and this indicates a one-step oxidation of ascorbic to dehydroascorbic acid.

The enzyme preparation was of such activity as to necessitate only a short incubation in the bath. Ascorbic acid was dissolved and neutralized a few seconds before being pipetted into the ice-cold Warburg flasks. Under these conditions the spontaneous breakdown of ascorbate was negligible and the blanks small.

Table 7. *Phosphorylation coupled with oxidation of reduced cytochrome* c

(The control system contained cytochrome *c*, 0·0001 M; L-ascorbate, 0·01 M, with other components as in Table 6. Temp. 15°. Rat-liver mitochondria in every case. The figures shown have had blanks subtracted as described in the text.)

System	O_2 uptake (μg.atoms)	P uptake from inorganic orthophosphate (μg.atoms)	P/O ratio	Time in bath (min.)
Control	8·0	7·3	0·91	7
10^{-4} M-DNP	8·9	0·0	—	7
Control	11·0	6·1	0·56	15
Control	9·0	4·5	0·50	10
Control	10·6	5·3	0·50	10

Table 8. *Oxidation of* L-*ascorbic acid by rat-liver mitochondria*

(Time in bath 12 min. Initial values determined by tipping acid into duplicate flasks at end of equilibration. All components as in Table 7. Temp. 15°. Gas phase: air.)

	Complete system (blank subtracted)	No cytochrome *c*	No enzyme	No enzyme, no cytochrome *c*
O_2 uptake (μg.atoms)	15·5	0·9	0·0	0·0
Ascorbic acid disappearing (μg.mol.)	15·3	0·0	2·5	0·0

Table 9. P/O *ratio of* β-*hydroxybutyrate oxidation*

(The control system contained 30–60 μg.mol. 1-β-hydroxybutyrate and 40 μg.mol. of inorganic orthophosphate. Other components as in Table 6. Rat-liver mitochondria. Temp. 15°. Gas phase: air.)

System	O_2 uptake (μg.atoms)	P uptake from inorganic orthophosphate (μg.atoms)	P/O ratio	Time in bath (min.)
Control	11·5	29·8	2·6	20
Control	8·1	23·4	2·9	28
DNP, 10^{-4} M	8·0	0·0	—	28

The results of these experiments do not support the hypothesis that the phosphorylation recorded above is due to the oxidation of breakdown products of dehydroascorbic acid; at least within the limits of accuracy of the methods.

Effect of L-*cysteine on* P *uptake.* When L-cysteine was the reducing agent, no P uptake was observed, confirming the finding of Lehninger (1949). However, the behaviour of cysteine was anomalous. The cytochrome oxidase activity was far smaller in the presence of this compound than in the presence of ascorbate; furthermore, though it had only a slight effect in diminishing oxidative phosphorylation in the presence of β-hydroxybutyrate, it severely impaired the P uptake when it was added together with ascorbate in the cytochrome oxidase system. No obvious explanation for these anomalies can be suggested.

Phosphorylation coupled with oxidation of reduced CoI*; effect of DNP.* Lehninger & Smith (1949) have shown that rat-liver particles oxidize β-hydroxybutyrate to acetoacetate, this oxidation being coupled with a phosphorylation, the P/O ratio being about 2·0. Lehninger (1949) has given reasons for regarding this phosphorylation as associated with the oxidation of reduced CoI. At 15° the oxidation of β-hydroxybutyrate gave a P/O ratio approaching 3·0 (Table 9). DNP abolishes the P uptake without affecting oxygen uptake. This point is considered again later.

Effect of DNP on an anaerobic dismutation of α-*ketoglutarate.* Hunter & Hixon (1949*b*) have shown that in the anaerobic dismutation of α-ketoglutarate and ammonia (in which 1 mol. of α-ketoglutarate is oxidized to succinate at the expense of a second mol. which is reduced to glutamate) a phosphorylation takes place, the value of P μg.atoms taken up per μg.mol. α-ketoglutarate oxidized approaching a value of 1·0. Repetition of these experiments at 15° confirmed this figure. Table 10 illustrates experiments in which the action of DNP and azide on this phosphorylation was tested. The inhibitory effect of DNP is relatively slight, azide has no effect on the P uptake, nor do the two compounds when added together. No effect on the dismutation was observed and the disappearance of ammonia is in good agreement with the requirements of the reaction. It should be noted that in Table 10 ratios are given as P/2 α-ketoglutarate; this follows from the fact that 2 mol. of substrate disappear for each one oxidized; the P uptake is therefore related to each pair of mol., that is, to one-half of the total α-ketoglutarate disappearing.

A phosphate balance study of the reaction mixture after incubation with DNP showed that the orthophosphate taken up was incorporated into an acid-stable ester which had a soluble barium salt, which was precipitated at pH 8·2 by the addition of 4 vol. of ethanol, and may therefore be presumed to be a hexosemonophosphate. No easily hydrolysable phosphate accumulated.

To confirm that the concentrations of azide used were effective under conditions in which they had been tested previously (Loomis & Lipmann, 1949), the anaerobic oxidation of glutamate and α-ketoglutarate by ferricyanide was studied. Table 11 shows that azide and DNP powerfully inhibit P uptake while leaving ferricyanide reduction unaffected. The phosphate uptakes with a number of substrates in the presence of ferricyanide are included in Table 11. The ratio P/2 ferricyanide approaches a value of 1·0 for all. This value is not exceeded at 15°. In Table 11 the last column shows the ratio μg.atoms P taken up per pair of μg.mol. ferricyanide reduced. This follows from the fact that a single electron is transferred per mol. of ferricyanide.

Table 10. *Phosphorylation in the anaerobic dismutation of α-ketoglutarate and ammonia. Action of DNP and sodium azide*

(The control system contained 30–50 μg.mol. α-ketoglutarate; 50–80 μg.mol. of NH_4Cl; 30 μg.mol. of orthophosphate and other components as in Table 6. The experiments were carried out in Thunberg tubes. Rat-liver mitochondria in every case. Temperature varied between 15 and 25° in different experiments.)

System	α-Ketoglutarate disappearing (μg.mol.)	NH_3 disappearing (μg.mol.)	P uptake from inorganic orthophosphate (μg.atoms)	P/2 α-ketoglutarate	Inhibition (%)
Control	22·0	10·0	6·5	0·59	—
10^{-4} M-DNP	22·0	9·0	6·5	0·59	0·0
2×10^{-3} M-NaN_3	22·0	11·0	6·5	0·59	0·0
Control	14·5	7·2	6·0	0·82	—
10^{-4} M-DNP	15·7	6·2	4·9	0·62	25·0
2×10^{-3} M-NaN_3	14·5	6·2	5·7	0·78	5·0
Control	30·0	—	9·4	0·63	—
2×10^{-4} M-DNP	30·0	—	6·0	0·40	36·0
4×10^{-3} M-NaN_3	30·0	—	9·4	0·63	0·0
Control	21·7	—	9·0	0·84	—
2×10^{-4} M-DNP	19·3	—	5·4	0·56	33·0
Control	27·5	—	13·0	0·95	—
2×10^{-4} M-DNP	25·6	—	8·7	0·68	28·0
8×10^{-3} M-NaN_3	22·5	—	9·6	0·85	9·0

Table 11. *Inhibition of* P *uptake by DNP and azide in presence of ferricyanide*

(The control system contained 30 μg.mol. of substrate in the case of glutamate, α-ketoglutarate and β-hydroxybutyrate and 100 μg.mol. in the case of succinate. Ferricyanide, 50 μg.mol., was added. Other components as in Table 6; rat-liver mitochondria in every case, the experiments being carried out in Thunberg tubes at temperatures varying between 15 and 25° in different experiments.)

System	Substrate	Ferricyanide reduced (μg.mol.)	P uptake from inorganic orthophosphate (μg.atoms)	P/2 ferricyanide
Control	Glutamate	16·8	4·9	0·58
NaN_3, 2×10^{-3} M	Glutamate	16·8	0·8	0·10
Control	Glutamate	20·5	8·1	0·79
Control	α-Ketoglutarate	25·0	11·3	0·90
DNP, 10^{-4} M	α-Ketoglutarate	25·0	0·0	—
NaN_3, 4×10^{-3} M	α-Ketoglutarate	25·0	1·3	0·14
Control	Succinate	34·8	7·4	0·43
Control	β-Hydroxybutyrate	22·4	9·9	0·89

As the anaerobic dismutation studied involves two oxidations:

$$\alpha\text{-ketoglutarate} \rightarrow \text{succinate}, \quad (1)$$
$$\text{Co I } H_2 \rightarrow \text{Co I}, \quad (2)$$

it is not possible to state precisely which reaction is coupled with the phosphorylation, though it is probably reaction (1). To obtain evidence on this point, α-ketoglutarate and β-hydroxybutyrate were incubated aerobically with rat-liver mitochondria in the presence of DNP. The purpose of this experiment was twofold: (1) to determine the effect of DNP on the oxidation of reduced Co I, as exemplified by the oxidation of β-hydroxybutyrate, and (2) to determine whether the aerobic oxidation of α-ketoglutarate would show a phosphate uptake in the presence of DNP, as suggested by the dismutation and contrary to previous experience (Cross *et al.* 1949). The inorganic orthophosphate addition was limited to 20 μg.mol. and the incubation times prolonged to make it possible to detect relatively small changes in orthophosphate content. Table 12 shows that no phosphate uptake was observed in the presence of β-hydroxybutyrate, while with α-ketoglutarate phosphorylation was easily demonstrable. It will be seen that while the P/O ratio for the latter substrate was 0·2, the ratio μg.atoms P taken up/μg.mol. α-ketoglutarate disappearing was 0·4, which is in fair agreement with the figures obtained in the dismutation in the presence of DNP. To confirm that the systems oxidizing the substrates were functioning properly, P/O ratios were determined in the absence of DNP. Figures of 3·4 and 2·9 were obtained for α-ketoglutarate and β-hydroxybutyrate respectively.

It thus appears likely that the phosphorylation in the anaerobic dismutation of α-ketoglutarate and ammonia is coupled with reaction (1) and that a mechanism exists whereby ATP can be formed in the presence of DNP. The site of action of the latter can also be inferred from these data; it is suggested as a working hypothesis that DNP inhibits phosphate

Table 12. *Phosphate uptake in presence of DNP in aerobic systems*

(The systems contained 30 μg.mol. α-ketoglutarate or 60 μg.mol. β-hydroxybutyrate, DNP, 2×10^{-4} M; inorganic orthophosphate, 20 μg.mol.; other components as in Table 6. Temp. 15°. Gas phase: air.)

Substrate	O_2 uptake (μg.atoms)	P uptake from inorganic orthophosphate (μg.atoms)	α-Ketoglutarate disappearing (μg.mol.)	P/α-ketoglutarate (μg.atoms/μg.mol.)
α-Ketoglutarate	21·0	4·7	12·0	0·40
	26·6	6·8	16·0	0·43
β-Hydroxybutyrate	16·0	Nil	—	—
	18·6	Nil	—	—
	16·7	Nil	—	—

Table 13. Co I *requirement for α-ketoglutarate oxidation in aged preparations of rat-liver mitochondria*

(The systems contained adenosine-5-phosphate, 0·001 M; inorganic orthophosphate, 0·01 M; $MgSO_4$, 0·0067 M; α-ketoglutarate, 0·01 M; KCl, 0·025 M; 0·5 ml. rat-liver mitochondria suspended in 0·25 M-sucrose and additions as indicated. Final volume 3·0 ml. Temp. 38°. Gas phase: air. For details of preparation, see text.)

		O_2 uptake (μl.)					
		Methylene blue					
Exp. no.	System	$0·8 \times 10^{-4}$ M	Nil	Cytochrome *c*, 10^{-5} M	No cytochrome *c*	No aneurin pyrophosphate	α-Ketoglutarate disappearing (μg.mol.)
1	Co I (5 μg.mol.)	233	235	—	—	—	—
	No Co I	33	26	—	—	—	—
2	Co I (5 μg.mol.) + malonate (0·03 M)	—	—	142	141	128	15·0
	Malonate (0·03 M), no Co I	—	—	65	70	—	7·5
3	Co I (5 μg.mol.)	—	—	280	—	—	18·0
	Alkali-inactivated Co I (5 μg.mol.)	—	—	80	—	—	12·0
	No Co I	—	—	98	—	—	12·0
	ATP (6·5 μg.mol.)	—	—	80	—	—	9·0

uptake in the respiratory sequence from pyridine nucleotide level upwards. Since no evidence exists that pyridine nucleotides play any part in the oxidation of α-ketoglutarate, it was decided to investigate this possibility.

Co I *requirement for α-ketoglutarate oxidation.* Rat-liver mitochondria were aged for 24–30 hr. after preparation. During this time they were maintained at 0°. On incubation after this period, with α-ketoglutarate as substrate, it was found that the oxygen uptake fell rapidly after 10 min. and soon became negligible. Table 13 shows that if Co I were added a great enhancement of respiration is observed. When the experiment is carried out in the presence of malonate, it will be seen that the changes are not so great, but an increased removal of α-ketoglutarate is confirmed by the chemical estimations. It can be seen that this increased removal amounts to 30–50%. The results in Table 13 also show that alkali-inactivated Co I is ineffective in stimulating the oxygen uptake, and that ATP will not replace pyridine nucleotide. There is no obvious requirement for aneurin pyrophosphate or cytochrome *c*; nor is methylene blue required as a carrier. When these aged preparations were tested for their ability to carry out oxidative phosphorylation, no activity could be detected in the experiments carried out at 38°. However, in a single experiment carried out at 25°, a P/O ratio of 1·5 was obtained with α-ketoglutarate as substrate; this experiment was conducted with a different preparation to those illustrated above.

In order to shorten the times of ageing, several experiments were done in which mitochondria were aged in the presence of DNP (2×10^{-4} M) for 1 hr. at 0°. In no case did this procedure have any effect on the ability of the particles to oxidize L-glutamate or α-ketoglutarate. The addition of large amounts of Co I did not increase oxygen consumption significantly, whether DNP were present in the flasks or not, and whether the systems were deficient in inorganic orthophosphate or not.

These experiments do not prove that the enzyme oxidizing α-ketoglutarate is a Co I linked dehydrogenase or a pyridinoprotein, in the sense that triosephosphate dehydrogenase may be considered one; Dr M. Dixon has pointed out that in mitochondrial systems the Co I may quite well be concerned in the regeneration of another co-factor essential for α-ketoglutarate oxidation.

The mechanism of phosphate 'replacement' by DNP

Loomis & Lipmann (1948) showed that in the absence of added inorganic orthophosphate, DNP caused a stimulation of respiration which raised the oxygen uptake of such deficient systems to the same value as that for the controls. This finding was confirmed by Judah & Williams-Ashman (1951). Teply (1949) claimed that pre-incubation of the particles with DNP destroyed this stimulating effect, and suggested that 'replacement' was due to liberation of inorganic orthophosphate from labile esters firmly bound to the particles (gel-P). This 'gel-P' could not be washed away with large volumes of 0·9 % (w/v) potassium chloride.

It has been found in the present work that washing of purified mitochondrial preparations with 0·25 M-sucrose reduces the phosphate concentration to a level where replacement is no longer observed. Figures for a typical experiment are presented in Table 14 and show that in the presence of α-ketoglutarate as substrate, and in a well washed preparation, DNP causes slight stimulation of respiration in the absence of added inorganic orthophosphate. The stimulating power of DNP may be increased by the addition of small amounts of inorganic orthophosphate. Figures are also given for initial and final inorganic orthophosphate levels in the flasks, and show that no output of inorganic orthophosphate has taken place. On the contrary, a marked uptake is seen, even in the presence of DNP. This could be expected from evidence presented earlier in this paper, which shows that DNP permits P uptake in α-ketoglutarate oxidation. Loss of 'replacement' by DNP could also be shown when L-glutamate was substrate. With β-hydroxybutyrate as substrate, the restoration of 'replacing' activity is achieved with much lower phosphate concentrations than is the case with α-ketoglutarate as substrate (Table 15) as might have been expected, as DNP completely blocks P uptake during this oxidation.

The reduction of stimulating ability could also be achieved when the oxidation of α-ketoglutarate and glutamate were carried out anaerobically in the presence of ferricyanide, but restoration was not satisfactory on addition of phosphate in small amounts. These results are presented in Table 16. They make it reasonable to doubt the hypothesis of Loomis & Lipmann (1949) that azide inhibits a transphosphorylation between a 'primary ester' and adenosine-5-phosphate, while DNP prevents the formation of 'primary ester'. This hypothesis was based on the inability of azide to 'replace' orthophosphate, a finding which has been confirmed.

Table 14. *Effect of DNP on oxidation of α-ketoglutarate in phosphate-deficient systems*

(The mitochondria were washed by five successive centrifugations at 15,000 *g* in 0·25 M-sucrose after an initial sedimentation at 9000 *g*. Substrate α-ketoglutarate, 0·01 M; other components as in Table 12, except that phosphate additions were as shown below. Temp. 25°. Rat liver in every case. Where phosphate was omitted NaCl was added to maintain salt concentration.)

Phosphate (μg.)			O_2 uptake (μl.)			
Initial	Final	Difference	1st 10 min.	2nd 10 min.	3rd 10 min.	DNP × 10^{-4} M
944	—	—	67	79	81	0·0
14	7·0	7·0	20	17	17	0·0
14	7·0	7·0	26	21	20	2·0
76	7·0	69·0	30	23	19	0·0
76	16·0	60·0	58	38	35	2·0
107	7·0	100	28	22	21	0·0
107	28·0	79	64	49	44	2·0
138	7·0	131	33	23	21	0·0
138	50·0	88	69	56	48	2·0

Table 15. *Effect of DNP on oxidation of* L-*glutamate and* 1-β-*hydroxybutyrate in phosphate-deficient system*

(Details as for Table 14, except for substrates. 30 μ.mol. L-glutamate or 30 μ.mol. 1-β-hydroxybutyrate added. Rat-liver mitochondria at 25°. Where phosphate was omitted NaCl was added to maintain salt concentration.)

Substrate	Phosphate (μg.)			O_2 uptake (μl.)	DNP × 10^{-4} M
	Initial	Final	Difference		
L-Glutamate	944	—	—	104	0·0
	14·0	14·0	0·0	29	0·0
	14·0	13·0	−1·0	42	2·0
1-β-Hydroxybutyrate	940	—	—	78	0·0
	10·0	—	—	13·0	0·0
	10·0	—	—	41·0	1·0
	950	—	—	51·0	0·0
	21·5	7·0	−14·5	24·0	0·0
	21·5	34·0	+12·5	40·0	1·0
	37·0	7·0	−30·0	24·0	0·0
	37·0	48·0	+11·0	46·0	1·0

Table 16. *Failure of DNP and azide to 'replace' P in presence of ferricyanide*

(In the first two experiments CO_2 output was determined manometrically; the flasks containing 0·02 M-$NaHCO_3$ with 95% N_2–5% CO_2 in the gas phase. The third experiment was carried out in Thunberg tubes. All components as in Table 11. 30 μg.mol. α-Ketoglutarate as substrate in Exps. 1 and 3; L-glutamate in Exp. 2; no hexokinase or fructose present. Rat-liver mitochondria. Temp. 25°. Where phosphate was omitted NaCl was added to maintain salt concentration.)

Exp. no.	System	Control	No orthophosphate added	No orthophosphate + DNP, 10^{-4} M	No orthophosphate + NaN_3, 8×10^{-3} M	No orthophosphate + DNP and NaN_3
1	CO_2 output (μl./4 min.)	253	147	168	160	181
2	CO_2 output (μl./10 min.)	187	128	157	—	—
3	Ferricyanide reduced (μg.mol./45 min.)	21·8	15·0	13·4	—	—

In order to explain the discrepancy between the present work and the findings of Teply (1949) several experiments were carried out. A lead to the correct interpretation was obtained by an experiment in which exhaustive washing of the mitochondria was not attempted. In this case it was found that sufficient phosphate remained in the preparation to show a typical replacement effect by DNP. However, when the particles were pre-incubated with DNP, this ability was lost. It is concluded that in the absence of proper washing, pre-incubation with DNP will reduce the potential inorganic orthophosphate. The source of this inorganic orthophosphate is not certain, but quite clearly there is no evidence to suggest 'gel-P' as being responsible. An experiment which suggests the contrary is shown in Table 17. This indicates that

Table 17. *Failure of DNP to 'replace' inorganic orthophosphate in the anaerobic dismutation of α-ketoglutarate and ammonia in phosphate-deficient systems*

(The reaction was followed manometrically. The complete system was as described in Table 10, but no fructose or hexokinase were present: in addition $NaHCO_3$, 0·02 M, was added. Gas phase, 95% N_2–5% CO_2. Temp. 25°. Rat-liver mitochondria. Where phosphate was omitted NaCl was added to maintain salt concentration.)

System	Complete	No added inorganic ortho-phosphate	No added inorganic ortho-phosphate + 10^{-4} M-DNP
CO_2 output (μl./5 min.)	102	15·0	28·0

the anaerobic dismutation of α-ketoglutarate and ammonia is very sensitive to phosphate deficiency. DNP did not 'replace', though 2·5 μg.mol. inorganic orthophosphate were present and the preparation had been subjected to only the normal washing procedure (two washes). As this dismutation is very sensitive to phosphate lack and as DNP affects the phosphorylation only slightly, it is clear that this experiment is a reasonable test for the hypothesis of Teply (1949) that DNP stimulation of phosphate-deficient systems is due only to liberation of inorganic orthophosphate from the particles.

It should be pointed out that exhaustive washing of mitochondria does not reduce their ability to esterify inorganic orthophosphate. Experiments on preparations subjected to six successive washings on the centrifuge at 15,000 ***g*** revealed that the efficiency of oxidative phosphorylation was unimpaired, P/O ratios of approx. 2·0 being obtained at 25°, this being the temperature at which all the experiments on phosphate replacement were carried out. From these experiments it is concluded that 'replacement' of inorganic orthophosphate by DNP represents a sparing or economizing of inorganic orthophosphate and that it is only manifest when a certain critical concentration of inorganic orthophosphate is present in the medium.

The synthesis of ATP from inorganic pyrophosphate and CoI

Judah & Williams-Ashman (1951) showed that inorganic pyrophosphate when added to respiring systems did not change in amount. However, it seemed certain that mitochondrial systems are capable of maintaining their coenzyme levels, and it was decided to investigate the possibility that the reaction described by Kornberg (1950) takes place in such systems. Kornberg showed that reversible synthesis of CoI can take place as follows:

$$\text{ATP} + \text{NMN} \rightleftharpoons \text{CoI} + \text{P.P},$$

where NMN is nicotinamide mononucleotide and P.P is inorganic pyrophosphate. The formation of ATP from CoI and inorganic pyrophosphate was readily demonstrated in rabbit-kidney mitochondrial systems. Table 18 illustrates an experiment in which hexokinase and fructose were added to the system, together with CoI and inorganic pyrophosphate. Under these conditions the terminal phosphate atom of ATP is removed as soon as formed and the

Table 18. *Synthesis of ATP from* Co I *and inorganic pyrophosphate*

(The complete system contained Co I, 0·001 M; inorganic pyrophosphate, 0·001 M; hexokinase, 0·02 ml.; fructose, 0·02 M; $MgSO_4$, 0·0067 M; NaF, 0·013 M; 0·5 ml. 0·1 M-veronal-HCl buffer; pH 7·40, and water to 3·0 ml. Temp. 38°. Time in bath 20 min. Rabbit-kidney mitochondria. All figures are corrected for small changes in orthophosphate content which occurred.)

System	Complete	No Co I	No inorganic pyrophosphate	No hexokinase
Decrease in easily hydrolysable P (μg.)	60	15	0·0	5·0

Table 19. *Phosphate balance of ATP synthesis from* Co I *and inorganic pyrophosphate*

(The system was as described in Table 18, but on five times the scale. The figures for easily hydrolysable P are corrected for orthophosphate.)

Barium-insoluble fraction

	Orthophosphate (μg.)	Easily hydrolysable P (μg.)	Total P (μg.)	Inorganic pyrophosphate (as orthophosphate) (μg.)
Initial values	440	920	1880	800
After incubation	360	360	1240	Nil
Difference	−80	−560	−640	−800

Barium-soluble: ethanol-insoluble fractions

	Orthophosphate (μg.)	Easily hydrolysable P (μg.)	Total P (μg.)	Reducing power in terms of fructose-6-P barium salt (mg.)
Initial values	Nil	Nil	1460	Nil
After incubation	Nil	Nil	820	9·5
Difference	—	—	−640	+9·5

Table 20. *Oxidative phosphorylation; pyruvate and octanoate oxidation after thyroxine treatment of rats*

(The mitochondria were prepared from equal amounts of tissue from control and thyroxine-treated rats (see text), except in the last experiment where no precautions were taken to obtain equal amounts. System as described in Tables 2 and 6. Temperature 38° except in the last experiment when it was 20°. 30 μg.mol. of substrate added except in the case of octanoate where 4 μg.mol. of fatty acid and 3 μg.mol. of succinate were used and in the case of pyruvate where 1 μg.mol. of succinate was added as primer to 30 μg.mol. of pyruvate.)

		Oxidative phosphorylation				
Mitochondria from	Substrate	O_2 uptake (μg.atoms)	P uptake from inorganic orthophosphate (μg.atoms)	P/O ratio	Pyruvate oxidation O_2 uptake (μl./20 min.)	Octanoate oxidation O_2 uptake (μl./20 min.)
Control	L-Malate	17·7	33·0	1·87	276	279
Thyroxine treated	L-Malate	21·4	26·6	1·24	395	393
Control	L-Malate	18·6	36·2	1·95	—	—
Control + 0·3 unit insulin	L-Malate	18·6	35·4	1·91	—	—
Thyroxine treated	L-Malate	22·1	36·2	1·65	—	—
Thyroxine treated + 0·3 unit insulin	L-Malate	22·2	36·2	1·64	—	—
Control	L-Glutamate	7·0	16·8	2·4	—	—
Thyroxine treated	L-Glutamate	6·5	13·0	2·0	—	—

reaction may be followed by the decline in readily hydrolysable phosphate (i.e. hydrolysable by incubation at 100° in N-hydrochloric acid for 10 min.). It will be seen that such a decline takes place in the presence of all components, but not in the absence of Co I, inorganic pyrophosphate or of hexokinase. Table 19 gives the result of a balance experiment carried out on a larger scale with an identical preparation; a satisfactory balance is obtained. The formation of ATP is indicated by the accumulation of hexose monophosphate as a result of the hexokinase reaction.

Further experiments showed that adenosine-5-phosphate will not substitute for Co I in this reaction.

It has been stated that no change in added inorganic pyrophosphate level is observed in freely respiring systems; however, if DNP (10^{-4} M) is added to a rabbit-kidney mitochondrial preparation while freely respiring, a decline in readily hydrolysable phosphate takes place when inorganic pyrophos-

phate is present. It may be inferred that during oxidative phosphorylation the maintenance of a high ATP level forces the reaction in the direction of Co I synthesis.

The action of thyroxine on oxidative phosphorylation. It has been shown that the addition of L-thyroxine or of thyroglobulin to systems *in vitro* affected neither their oxygen uptake nor the esterification of inorganic orthophosphate (Judah & Williams-Ashman, 1951). Mr E. V. Rowsell, of the School of Biochemistry, Cambridge, has kindly informed me of unpublished experiments in which he observed a diminution of the P/O ratio during β-hydroxybutyrate oxidation by particles prepared from rat livers after the rats had been rendered hyperthyroid by thyroxine injections.

These experiments were therefore repeated. All animals used in this work were litter mates and were maintained on high-vitamin diets; food intake was controlled by pair-feeding. The animals were treated with DL-thyroxine, injected subcutaneously in 1·0 mg. doses every other day. The controls were injected with solvent. After seven consecutive injections, the animals were killed and mitochondria prepared from their livers in the usual manner. The results in Table 20 show that the effect of thyroxine on oxidative phosphorylation was not considerable under these conditions, and that the effect on fatty acid oxidation was the reverse of that of DNP. The oxidation of pyruvate is also enhanced by thyroxine treatment. It is of some interest that turbidimetric determination of the number of mitochondria and estimation of ribonucleic acid phosphate showed that equal numbers of particles were present in the flasks containing control and thyroxine-treated tissue. These experiments were not extended, but they indicate that the action of thyroxine both *in vitro* and *in vivo* is essentially different from that of DNP.

DISCUSSION

From the experiments quoted in this paper, the site of action of DNP can be inferred. It has been shown that DNP only slightly reduces the phosphate uptake coupled with the anaerobic dismutation of α-ketoglutarate and ammonia; it has also been shown that in the aerobic oxidation of the same substrate in the presence of DNP, a phosphate:α-ketoglutarate ratio approximating to that obtained in the dismutation is found. Since DNP causes complete abolition of phosphate uptake associated with β-hydroxybutyrate oxidation, it seems reasonable in the light of present knowledge to assume that DNP inhibits phosphate uptake in the respiratory sequence from pyridine nucleotide upwards. In making this suggestion, it is assumed that Co I takes part in the oxidation of α-ketoglutarate. Though this has never been shown conclusively, evidence is presented in this paper that it may in fact participate. It has also been assumed that the oxidation of reduced Co I (as exemplified by the oxidation of β-hydroxybutyrate) involves an identical mechanism whatever the substrate. There is no evidence for this, but it has been accepted for want of evidence to the contrary. It is obvious that these assumptions must make the conclusion tentative.

It seems clear that DNP does not uncouple oxidation from phosphorylation in the sense that arsenate uncouples these processes in the oxidation of triosephosphate. (Mr E. V. Rowsell has kindly informed me of experiments conducted by Dr G. D. Greville and himself (1949, unpublished observations) in which it was found that DNP has no effect on phosphorylation coupled with the anaerobic oxidation of triosephosphate, using pyruvate as hydrogen acceptor and muscle acetone powder as source of enzyme.) While no proof is forthcoming, it is suggested that DNP inhibits a transphosphorylation between an unstable ester or esters and adenosine-5-phosphate at the three successive steps from pyridine nucleotide, through flavoprotein to the cytochrome system. Some evidence is available that DNP may act in the manner indicated. It is clear that the methods normally used to estimate inorganic orthophosphate (deproteinization with trichloroacetic acid followed by an estimation of the molybdenum blue colour in strongly acid solution) might well fail to demonstrate the presence of a very labile phosphate compound. Some experiments were undertaken to test this point. Deproteinization was carried out by half-saturation with ammonium sulphate, followed by estimation of inorganic orthophosphate by the method of Lowry & Lopez (1946). When this method was applied to systems poisoned with DNP, it was found that colour development was delayed some 15 min. beyond the time required in the control and initial estimations. This suggested the presence and breakdown of an unstable ester. Extrapolation to zero time gave values for P/O ratio of approx. 2·0. The requisite control estimations indicated that no simple artifact was involved. Unfortunately, such results were not reproducible. Numerous attempts were made to precipitate inorganic orthophosphate under the mildest conditions in the hope that they might show the presence of the suspected labile compounds, but were not successful. Unfortunately, this work has to be suspended for the time being, and the preliminary observations given in this paper are recorded in the hope that further work may be stimulated.

The confirmation of Hunter & Hixon's (1949*a*) observations on the P/O ratio of α-ketoglutarate oxidation needs further comment. No attempt has been made to correct the ratios obtained in this work, though Hunter has introduced corrections which tend to lower his high initial figures. It would appear

that such corrections may well be necessary and may be exemplified by the case of the figures obtained for glutamate oxidation. In the latter, P/O ratios of 3·0 and over have regularly been found. Now the E'_0 of the glutamate-α-ketoglutarate system has been given as −0·03 V. at pH 7·0 by Kalckar (1941), and assuming a value of 12,000 cal. for the high-energy phosphate bond of ATP at pH 7·0 (Lipmann, 1941), this only gives room for a P/O ratio of 3·0, if one accepts the observed value of 1·0 for the P/O ratio for electron transport over the cytochrome system (cf. Dixon, 1949). Furthermore, the oxidation of β-hydroxybutyrate gives a P/O ratio of 3·0, this system having E'_0 of −0·293 V. at pH 7·0, while that of Co I is given as −0·281 V. It is therefore difficult to account for a P/O ratio of 4·0 for the oxidation of glutamate to α-ketoglutarate.

For the latter substrate, however, a case can be made for a P/O ratio as high as 4·0. The phosphorylations can be apportioned as follows: (1) The oxidation of α-ketoglutarate to succinate yields one phosphorylation; the evidence for this is presented in the present work. (2) In the presence of ferricyanide, α-ketoglutarate yields P/2 ferricyanide approaching 1·0. This phosphorylation cannot be that associated with (1) above, because it is shown that both azide and DNP inhibit in the presence of ferricyanide, but do not affect (1). It would seem that ferricyanide itself must inhibit at least one phosphorylation, which is most likely that associated with (1) above. (3) and (4) In the aerobic oxidation of β-hydroxybutyrate, a P/O ratio approaching 3·0 is observed, and if it is accepted that the oxidation of reduced cytochrome *c* gives a P/O ratio of 1·0, and it has been shown that β-hydroxybutyrate gives a P/2 ferricyanide of 1·0, the third phosphorylation probably occurs at flavoprotein level, assuming a value of 12,000 cal. for the high-energy phosphate bond. Thus, the third and fourth phosphorylations in α-ketoglutarate oxidation would be due to oxidations of reduced flavoprotein and cytochrome respectively. It is obvious that the nature of the assumptions made in this argument is such that the conclusions are open to the gravest doubts. However, it has been considered worth while making these points to formulate a working hypothesis which has the merit of linking together a series of isolated observations. The P/O ratio for β-hydroxybutyrate oxidation has been accepted in this argument because it is known that rat-liver particles oxidize this substance quantitatively to acetoacetic acid, and side reactions cannot contribute to a falsely high ratio.

Hummel & Lindberg (1949) have demonstrated that when ^{32}P is incubated with rabbit-kidney particles carrying out oxidative phosphorylation the indicator is incorporated into flavine-adenine dinucleotide (FAD) and ATP but not into Co I. Though these results are suggestive they are by no means conclusive; thus the marker will not enter Co I if its synthesis follows the reaction described by Kornberg (1950), unless the ^{32}P has been incorporated into the phosphate group of adenosine-5-phosphate. Similarly, the incorporation of the label into FAD may simply indicate that the mechanism of synthesis of the latter does not follow an identical course to that described by Schrecker & Kornberg (1950).

Evidence is presented in this paper that the reaction between Co I and inorganic pyrophosphate does occur in rabbit-kidney mitochondria, and since it is reasonable to suppose that this reaction is reversible (as is that described by Kornberg), a mechanism exists for the synthesis of Co I in these systems. It is suggested that mitochondrial systems can maintain their own coenzyme levels so long as they are capable of oxidative phosphorylation. The observations recorded in this paper on the reversal of DNP inhibition of pyruvate oxidation and on the coenzyme requirements of aged preparations support this view. Such a hypothesis would also explain the fact that the only known coenzyme requirement of freshly prepared particles is that for adenosine-5-phosphate. There is therefore no need to postulate the existence of a new type of adenyloprotein (Cross *et al.* 1949). Furthermore, the formation of inorganic pyrophosphate may be readily explained without suggesting the breakdown of hypothetical 'coenzyme pyrophosphates' (Cross *et al.* 1949).

Much of the evidence given above is unsatisfactory. It has been assumed that α-ketoglutarate oxidation involves pyridine nucleotide, but the demonstration of Co I requirement in this oxidation leaves open the possibility that Co I is playing an indirect part. The coupling of a phosphorylation with the oxidation of reduced cytochrome *c* is by no means certain. The experiments presented in this paper show that it is difficult to explain a phosphate uptake in the presence of cytochrome *c* and ascorbate as being due to the further breakdown of dehydroascorbic acid; on the other hand, the absence of effect in the presence of cysteine is an anomaly, further complicated by the fact that while cysteine has no effect on the overall P/O ratio of β-hydroxybutyrate oxidation, it certainly reduces the phosphate uptake when it is present together with ascorbate. The problem of this phosphorylation must be solved by a direct experiment. Indirect methods which attempt to bypass the cytochrome system are open to several criticisms. Friedkin & Lehninger (1949), who attempted to analyse the oxidative phosphorylation system, used carriers such as methylene blue and brilliant cresyl blue, but were unable to find any phosphate uptake in so doing. Judah & Williams-Ashman (1951) showed that both these dyes inhibit

oxidative phosphorylation. Friedkin & Lehninger (1949) also attempted to show a phosphate uptake under anaerobic conditions when excess cytochrome *c* was used as hydrogen acceptor. They failed to show any phosphorylation. Slater (1950) has recently repeated these experiments using α-ketoglutarate as substrate and has obtained P/O ratios which appear to be exactly the same whether cytochrome *c* was used as acceptor anaerobically or the system was allowed free access to oxygen. The source of enzyme in his experiments was a mitochondrial preparation isolated from cat heart. An experiment of this type is open to an obvious fallacy when it is used to determine the presence or absence of phosphorylation through the cytochrome system. As the two efficiencies have to be compared, it has to be assumed that each one represents a near approach to a maximum value. That is, a value between 2·0 and 2·9 can be taken as approaching a value of 3·0, and so on. Therefore, fractional differences between the ratios may be missed. However, this simplification is not justified; to take an extreme example it can be supposed that a series of four steps yield phosphorylations, the maximum possible P/O ratio being 4·0. Leaks and side reactions may result in 50% efficiency at each step, so that the observed P/O ratio is near 2·0. If it is a little below, it will be taken as 2·0, if a little above, it will be taken as 3·0. A greater possibility of error arises when one step is more liable to inhibition or fractional efficiency than the others. In this case, the increment due to the step in question may easily be overlooked, whereas if a direct determination were made, such an increment would be readily recognized. It seems reasonable to assume, therefore, that overall values for P/O ratios are not of great value in deciding the possible number of phosphorylations. An example which supports this belief can be quoted from determinations made in the present work and those reported in a previous paper (Judah & Williams-Ashman, 1951) for the oxidation of β-hydroxybutyrate by mitochondria. In the earlier determinations values for the P/O ratio of just over 1·0 were observed in experiments at 38°, and we believed that the maximum ratio was 2·0; at a bath temperature of 15°, values of well over 2·0 have been found, and it appears that a maximum value of 3·0 is not unreasonable. The preparations of enzyme and other components were identical in the two investigations.

As the present work has covered much the same ground as that of Friedkin & Lehninger (1949), though by different means, their results are of considerable interest in this discussion. Using ^{32}P as an indicator, these workers investigated the phosphate uptake associated with the oxidation of reduced Coi. In contrast to the work of Ochoa (1943), they found a marked incorporation of the label into ATP during the oxidation; though the results cannot supply an index of the P/O ratio, it was obvious that dihydrocozymase oxidation gave rise to as great an esterification as did the oxidation of malate. They explained the lack of phosphate uptake in Ochoa's experiments as being due to the high concentrations of nucleotide used by the latter. These have been found to be inhibitory, and it is suggested that the inhibition is not due to heavy metal contaminants but to the nucleotide itself. In order to facilitate analysis, Lehninger (1949) then used β-hydroxybutyrate as substrate, arguing from the standard oxidation-reduction potentials of this system and that of Coi that a phosphorylation at substrate level was unlikely, and that β-hydroxybutyrate oxidation represented to all intents an oxidation of reduced Coi. Indirect experiments with a dismutation between oxaloacetate and β-hydroxybutyrate and direct experiments where excess Coi was used as hydrogen acceptor supported this view. It has been confirmed in this laboratory that the anaerobic oxidation-reduction of β-hydroxybutyrate and Coi does not give rise to a phosphate uptake, and the new experiments dispose of a possible criticism of Lehninger's experiment that in the absence of sodium fluoride, hexokinase and a hexose acceptor, the small phosphate exchange would not be observable. Nevertheless, it cannot be regarded as certain that there is no phosphate uptake coupled to the oxidation of β-hydroxybutyrate to acetoacetate. It should be mentioned that the latter substance is not further oxidized by rat-liver particles. Lehninger followed these observations by an attempted analysis of the steps at which phosphorylation might occur. Despite the pronounced phosphate uptake in the whole system, he was unable to show any uptake when the cytochrome system was by-passed. The use of carriers such as methylene blue has already been mentioned, but it was also impossible to show any phosphate uptake when ferricyanide was used as hydrogen acceptor.

This observation has not been confirmed in the present work, and it is suggested that the earlier failure was due to the fact that no fluoride and no hexokinase and hexose were added; with the low ratios found in the presence of ferricyanide, there is no doubt that these omissions would have a decisive effect.

Experiments in which excess cytochrome *c* was added as hydrogen acceptor under anaerobic conditions have already been referred to. These were negative probably for the same reasons as the ferricyanide experiments. These experiments of Friedkin & Lehninger (1949) and of Lehninger (1949) clearly showed that while the over-all oxidation of reduced Coi gave rise to a well marked phosphate uptake, any attempt under their conditions to demonstrate the participation of the partial reactions was negative; later work has equally well shown that some of these

defects could be remedied, and the results underline the comments made earlier with regard to the differing efficiencies of the whole system and its parts.

The results obtained in the present work on the stimulation of phosphate-deficient systems provide reasonable evidence that DNP does not substitute for inorganic orthophosphate in these systems. This, in effect, confirms the view of Teply (1949); on the other hand, the suggestion of the latter that DNP exerts this effect by discharging 'gel-P' is not supported. It has been found that reduction of the residual inorganic orthophosphate to about 0·5 μg.mol./vol. of particles added to the flasks is sufficient to abolish the 'replacement' effect with all the substrates tested. When α-ketoglutarate is the substrate, the amount of phosphate required for full replacement is higher than when β-hydroxybutyrate or L-glutamate are used. Examination of the protocols of Teply (1949) reveals that the concentrations of 'gel-P' that he found are probably insufficient to account for DNP action. Thus he quotes the following values for 'gel-P': initial values of 0·55 μg.mol. for an untreated suspension and 0·09 μg.mol. for a DNP pre-incubated suspension. After incubation with substrate and other components, the 'gel-P' dropped to a level of 0·04 μg.mol. in the presence of DNP in phosphate-deficient systems, and rose to a value of 0·15 μg.mol. in phosphate-deficient systems in the absence of DNP. In the complete system the final value was 0·49 μg.mol. No values are recorded for complete systems in the presence of DNP, nor are inorganic phosphate levels in the suspension media. The substrate was α-ketoglutarate in this experiment. While it is possible that differences in preparations might account for differences in phosphate requirement, it is suggested that gross variations are unlikely. In the absence of figures for inorganic orthophosphate actually present, rather than values for 'gel-P', it is suggested that Teply's hypothesis is doubtful.

The significance of the 'gel-P' described by Green, Atchley, Nordmann & Teply (1949) is also doubtful. Though these workers provide evidence that 'cyclophorase' preparations become radioactive upon incubation with ^{32}P and that this phenomenon is associated with oxidative processes, there is no evidence whatever that the tracer has really been incorporated into the particles, whether in a labile coenzyme-linkage or not. Thus, 'gel-P' might well represent an ester or esters formed (free) in the suspension medium, and difficult to remove from the particles. There is nothing in the inability to wash the particles free of this material to suggest that it is actually incorporated into them. It is difficult to wash out all traces of substrate from such preparations also, as has been found both in the present work and by Green *et al.* (1948). Another point which has not been satisfactorily established is whether the 'gel-P' is actually concerned with the active particles and not with the debris associated with 'cyclophorase' preparations. Though an experiment is quoted in which it is claimed that 'gel-P' is found in mitochondrial preparations, an examination of the protocols reveals that the tissue was homogenized in a Waring blender, a process known both to damage the mitochondria and to result in nuclear disintegration (Potter, 1950). Furthermore, it is not at all clear from this experiment to what washing procedure the mitochondria had been subjected; nor is there any cytological evidence to show that the suspension was indeed one of mitochondria alone. This is a precaution which has been taken regularly in this laboratory. These experiments must, therefore, be accepted with reserve, a situation common to many experiments with particle systems, whether purified cytologically or not.

A solution to many of the problems discussed in this paper must await the purification of the multienzyme systems concerned. A start in this direction has already been made, but the temporary suspension of this work has made a detailed investigation impossible. It has been found that soluble proteins may be obtained from isolated mitochondria by a simple method. Treatment of the particles with 5 vol. ethanol at −20° for 30 min. resulted in their complete precipitation. The particles were recovered by centrifugation in the cold and then extracted with 0·1 M-phosphate buffer, pH 7·4. The cloudy extract contained much soluble protein. Ammonium sulphate fractionation of the crude extract yielded a soluble glutamic dehydrogenase, which rapidly decolorized methylene blue in the presence of L-glutamic acid and Co I. It has, so far, proved impossible to obtain a cytochrome oxidase in solution, nor have tests so far carried out revealed the presence of the phosphokinases concerned in oxidative phosphorylation. These observations are recorded in the hope that improvements of the method may result in the purification of these important systems. It should, at all events, be clear that the enzymes of insoluble particles are by no means insusceptible to ordinary methods of isolation, and this justifies the hope that the fundamental problems of oxidative phosphorylation may be solved in the near future by a direct approach, rather than by the doubtful methods hitherto employed.

SUMMARY

1. Mitochondrial systems catalysing the complete oxidation of pyruvate have been described.

2. The mechanism of the inhibition of pyruvate oxidation by 2:4-dinitrophenol has been investigated; the reversal of such inhibition has been demonstrated. Adenosinetriphosphate, coenzyme I and added dicarboxylic acids of the citric acid cycle will bring about this reversal.

3. Phosphorus/oxygen ratios for pyruvate, α-ketoglutarate, L-glutamate and β-hydroxybutyrate have been determined at 15°. Values approaching 4·0 have been obtained for the first three substrates and approaching 3·0 for the fourth. The significance of these findings has been criticized and discussed. A requirement for coenzyme I in α-ketoglutarate oxidation has been indicated, but not proved conclusively.

4. The phosphorylation coupled with the anaerobic dismutation of α-ketoglutarate and ammonia has been investigated. It is shown that DNP and azide scarcely affect this phosphorylation.

5. A phosphorylation has been shown to accompany the oxidation of reduced cytochrome *c* by rat-liver mitochondria in the presence of ascorbic acid. Anomalous results have been obtained in the presence of cysteine; the existence of a phosphorylation in the oxidation of reduced cytochrome *c* cannot be regarded as finally established.

6. Phosphorylation during anaerobic oxidation of a variety of substrates in the presence of ferricyanide has been investigated.

7. A site of action is proposed for 2:4-dinitrophenol. It is suggested that this involves all trans-phosphorylations from pyridine nucleotide level upwards.

8. The 'replacement' of inorganic orthophosphate by 2:4-dinitrophenol has been reinvestigated. In the case of purified mitochondria, there is no need to postulate that the breakdown of labile esters plays any part in the 'replacement'. It is suggested that 'replacement' is the consequence of a sparing of inorganic orthophosphate and evidence is presented to support this hypothesis.

9. The synthesis of adenosinetriphosphate from coenzyme I and inorganic pyrophosphate has been demonstrated in mitochondrial systems.

10. The action of thyroxine on oxidative phosphorylation, pyruvate oxidation and fatty acid oxidation has been investigated. This action is entirely different from that of 2:4-dinitrophenol, both *in vivo* and *in vitro*.

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The Catalytic Effect of Molybdate on the Hydrolysis of Organic Phosphate Bonds

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The great instability of creatine phosphate (Fiske & Subbarow, 1929), acetyl phosphate (Lipmann & Tuttle, 1944) and 1:3-diphosphoglyceric acid (Negelein & Brömel, 1939) in solutions containing molybdate has been known almost as long as these compounds themselves. It could not fail to attract the attention of the analyst who used a method of phosphate estimation of the Briggs (1922) or Fiske & Subbarow (1925) type, because these methods entail a period of colour development during which the organic phosphate is in contact with ammonium molybdate concentrations of 0·2–0·7 %.

The acid-lability of a phosphate bond is apparently not invariably linked with the catalytic effect of molybdate. For ribose-1-phosphate, for example, which is intermediate in acid-stability between creatine phosphate and acetyl phosphate, Kalckar (1947) found, in dilute acids, only about 30 % increase of the hydrolysis rate in presence of molybdate, and Meyerhof & Lohmann (1928) even made the curious observation that the hydrolysis of arginine phosphate in acid solution was actually inhibited in presence of molybdate.

It is perhaps surprising that, apart from these scattered observations, no systematic study of the molybdate effect has yet been made. To some extent this may be due to the fact that with the older methods of phosphate estimation a tedious separation of organic and inorganic phosphate was necessary for its demonstration. In the extraction method of phosphate estimation introduced by Berenblum & Chain (1938) the time of contact between molybdate and phosphate before completion of the phosphomolybdate extraction can be reduced to about 15 sec. This time interval is sufficiently short to reduce the interaction between molybdate and all but the most labile phosphate bonds to insignificant proportions. The method of Berenblum & Chain is, however, somewhat laborious and unsuited to the handling of large series of analyses. The recent modification of Martin & Doty (1949) has greatly simplified it, and with the adaptations which we used in the present study the method is as easy and expeditious to carry out as the method of Fiske & Subbarow without sacrificing any of the advantages of Berenblum & Chain's method.

METHODS

Estimation of phosphate. For the purpose of this investigation the procedure of Martin & Doty (1949) has been modified as follows: (1) the addition of the silicotungstate reagent was omitted since no protein was present in the solutions to be analysed; (2) an economy in solvents and an increase in sensitivity was obtained by reducing the scale of the method; (3) pure *iso*butanol was used instead of the 1:1 mixture of *iso*butanol and benzene recommended by Martin & Doty. Excellent reproducibility of results was obtained and the method has given complete satisfaction.

Solutions

5 % *Ammonium molybdate* in 4 N-H_2SO_4 (for inorganic P).

5 % *Ammonium molybdate* in water (for digests of total organic P, containing 1 ml. 60 % perchloric acid).

Stannous chloride. Stock solution: 10 g. $SnCl_2$ in 25 ml. conc. HCl.

Stannous chloride. Dilute: 0·5 ml. concentrated solution, diluted to 20 ml. with N-H_2SO_4. Prepare fresh daily.

Acid ethanol. 10 ml. conc. H_2SO_4 + 490 ml. absolute ethanol.

Procedure

Add 6 ml. *iso*butanol and 1 ml. acid ammonium molybdate solution to 5 ml. of a test solution containing 2–25 μg. inorganic P and shake for 15 sec. in a glass-stoppered test tube. With a fine-tipped rubber-teat pipette draw off the *iso*butanol layer as completely as possible, carefully avoiding any admixture of the aqueous phase. Transfer the *iso*butanol extract to a second test tube containing a pinch of anhydrous Na_2SO_4 and shake until the extract is cleared of any emulsified droplets. Withdraw 5 ml. and transfer to a third test tube. Add 5 ml. acid ethanol, 0·2 ml. dilute $SnCl_2$ solution, mix and measure the absorption with a red filter (e.g. Ilford spectrum 608). Construct a calibration curve in the usual way.

Measurement of the molybdate effect. In the majority of experiments a final concentration of 1 % ammonium molybdate was used. The test solution was made up of 3 parts buffer, 1 part 5 % aqueous molybdate and 1 part solution of organic phosphate compound, the latter usually in a concentration of 2 or 4 mM (final concn. 0·4 or 0·8 mM). Buffer and phosphate solutions on the one hand and molybdate solution on the other were separately adjusted to the required temperature and mixed at time t_0. Samples of 1–5 ml. were withdrawn at times t_1, t_2, etc., and, if necessary, cooled to room temperature. If the sample withdrawn for analysis amounted to y ml., the volume of 5 % molybdate in 4 N-H_2SO_4 was reduced to 1–0·2y ml. and 0·2y ml. of 4 N-

H_2SO_4 was added instead. In this way the molybdate and acid concentrations used for the phosphate analysis were kept constant.

Control solutions contained 1 part of water instead of molybdate solution. Care was taken to reduce to a minimum the time of contact with molybdate during the extraction by rapid addition of the molybdate solution after all other components, including *iso*butanol, had been added.

Higher concentrations of molybdate were used in some experiments. Owing to the difficulty of buffering these solutions they were only used when strict pH control was not required and when the experiment could be carried out in the neighbourhood of pH 5. The addition of molybdate solution for phosphate analysis was again so adjusted as to reach the standard concentration.

Procedure in presence of acetyl phosphate. Acetyl phosphate is so labile in presence of molybdate that the separation method must be employed. At time *t* 1 ml. of the test solution was withdrawn and immediately delivered into a previously determined amount of alkali (or acid) sufficient to bring the pH of the mixture to 7·0. Inorganic phosphate was then precipitated with ethanolic $CaCl_2$ according to Lipmann & Tuttle (1944).

Buffering. With an ammonium molybdate concentration of 1% the following buffers were found suitable:

pH 1–6: 0·2M-sodium acetate-HCl and sodium acetate-acetic acid buffers (Walpole, 1914).

pH 6–9: 0·2M-sodium borate-HCl and sodium borate-NaOH buffers (Sørensen, 1912).

Values below pH 1–1·5 were obtained by the addition of HCl to a final concentration of 0·5–1·0N.

The final pH of the mixture was measured in every case with a glass electrode standardized against 0·05M-potassium biphthalate (pH 4·00; Marconi buffer solution tablets).

Preparations

Barium adenosinetriphosphate (ATP). Prepared from rabbit muscle by the method of Dounce, Rothstein, Beyer, Meier & Freer (1948). Total organic P, 11·65%; acid-labile P 7·82% (hydrolysed in N-HCl at 100° for 7 min.).

Creatine phosphate. Synthesized according to Zeile & Fawaz (1938). The Ca salt contained 7·68% acid-labile P (purity of 80%).

Acetyl phosphate. Synthesized according to Lipmann & Tuttle (1944, 1947). The crystalline Ag salt contained 8·15% acid-labile P.

Phospho-enol-pyruvic acid. Synthesized according to Baer & Fischer (1949). The Ba salt contained 7·64% iodine-labile P.

Glucose-1-phosphate. Synthesized according to Krahl & Cori (1949). The Ba salt contained 6·68% acid-labile P.

Glucose-6-phosphate. Synthesized according to Lardy & Fischer (1946). The Ca salt, dried *in vacuo* at 78°, contained 10·69% organic P.

Fructose-6-phosphate. Prepared from hexosediphosphate by the method of Neuberg, Lustig & Rothenberg (1943). The Ba salt contained 6·43% organic P.

Fructose-1:6-diphosphate. The acid Ba salt, prepared from a commercial sample by the method of Neuberg *et al.* (1943), contained 12·80% organic P.

Sodium pyrophosphate and sodium α-glycerophosphate were commercial samples.

RESULTS

Effect of molybdate concentration. The catalytic effect of molybdate increases with increasing concentration, although the increase is not strictly linear, especially at higher concentrations (Fig. 1).

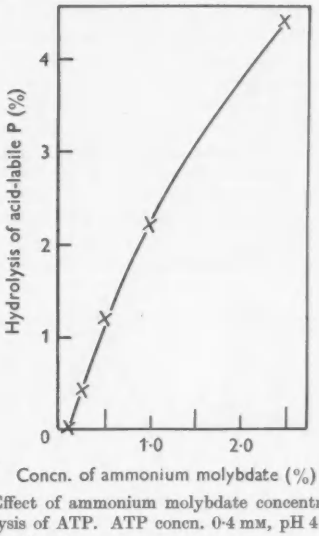


Fig. 1. Effect of ammonium molybdate concentration on hydrolysis of ATP. ATP concn. 0·4 mM, pH 4·1, temp. 30°, time 30 min.

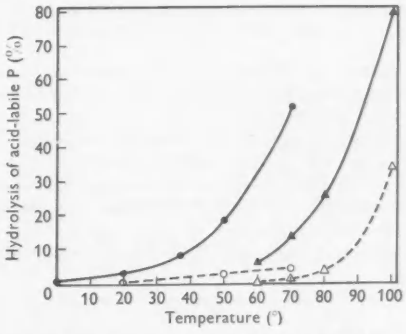


Fig. 2. Effect of temperature on the hydrolysis of ATP and sodium pyrophosphate in presence and in absence of ammonium molybdate. Concentration: substrates 0·4 mM, ammonium molybdate 2·5%; pH 5·2, 30 min. Hydrolysis in presence of ammonium molybdate, ——; controls, – – –; ATP, ○ and ●; pyrophosphate, △ and ▲.

On the other hand, a tenfold increase of substrate concentration did not change the percentage hydrolysis. The facts suggest that the catalytic effect depends on the formation of an unstable association complex. As long as molybdate is

present in great excess, an increase of substrate concentration will not affect the ratio of dissociated to undissociated complex. On the other hand, an increase in the concentration of molybdate will favour the formation of the complex.

Effect of temperature. The temperature-activity curve has a typical exponential form (Fig. 2). From the data in Fig. 2 a temperature coefficient $d \ln k/dT$ of 0·075 has been calculated for the first hydrolysis constant of ATP (t in min.).

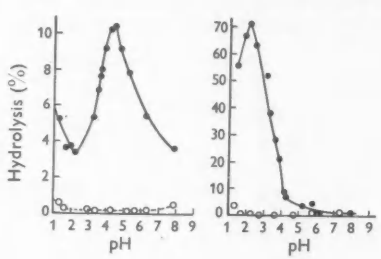
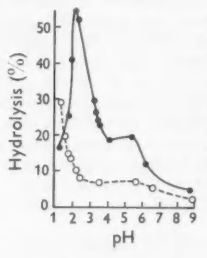


Fig. 3.

Fig. 4.



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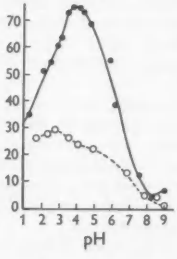

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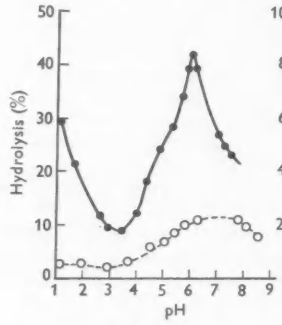

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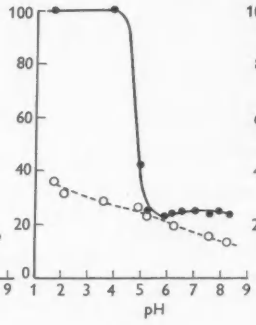

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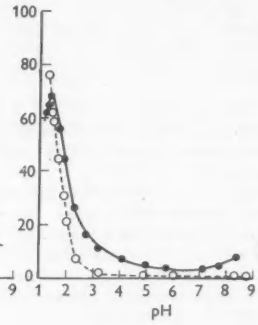

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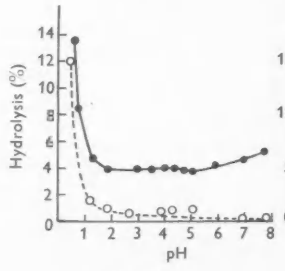

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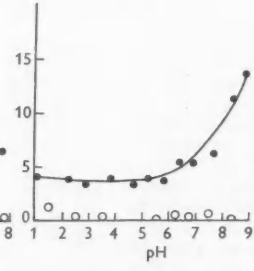

Fig. 11.

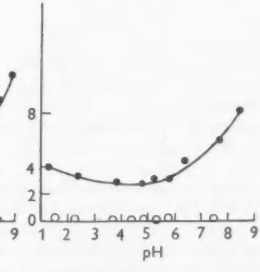

Fig. 12.

Figs. 3–12. Effect of pH on the hydrolysis of phosphate bonds in presence and in absence of ammonium molybdate. ●—●—●, hydrolysis in presence of 1% ammonium molybdate. ○--○--○, control without ammonium molybdate. Fig. 3. ATP (0·4 mM), 35°, 30 min. Fig. 4. Creatine phosphate (0·8 mM), 0°, 30 min. Fig. 5. Sodium pyrophosphate (0·4 mM), 80°, 30 min. Fig. 6. Phospho-enol-pyruvate (0·8 mM), 80°, 30 min. Fig. 7. Fructose-6-phosphate (0·8 mM), 80°, 120 min. Fig. 8. Acetyl phosphate (0·8 mM), 0°, 15 min. Fig. 9. Glucose-1-phosphate (0·8 mM), 70°, 30 min. Fig. 10. Fructose-1:6-diphosphate (0·8 mM), 70°, 30 min. Fig. 11. α-Glycerophosphate (0·8 mM), 80°, 120 min. Fig. 12. Glucose-6-phosphate (0·8 mM), 80°, 120 min.

Effect of pH. The influence of pH on the molybdate effect is illustrated in Figs. 3–12 for a number of organic phosphates. The temperatures and times used in the different experiments were so chosen for

convenience that a significant hydrolysis took place. In at least one case (pyrophosphate) where the effect was studied at 50, 70 and 80° the position of the pH optimum was unaffected by temperature. The effect has frequently a well pronounced pH optimum which varies from compound to compound. Some of the curves even show indications of two peaks, an 'alkaline' and an 'acid' pH optimum, as in the case of fructose-6-phosphate (Fig. 7), ATP (Fig. 3) and pyrophosphate (Fig. 5). With two of the compounds, pyrophosphate (Fig. 5) and glucose-1-phosphate (Fig. 9), the hydrolysis curves in presence and in absence of molybdate cross each other in the acid region of the pH scale. Here molybdate inhibits the hydrolysis, as has been found by Meyerhof & Lohmann (1928) for arginine phosphate.

Glucose-6-phosphate (Fig. 12) and α-glycerophosphate (Fig. 11) show a flat curve with a rise on the alkaline side of neutrality. The curve of glucose-1-phosphate is similar; the rise in the acid range is closely followed, and eventually overtaken, by the curve of the uncatalysed hydrolysis.

Table 1. *Hydrolysis of glucose-1-phosphate in presence of* 1 % *ammonium molybdate*

(9 ml. 0·2 M-acetate-HCl buffer, pH 2·6, 3 ml. 5% ammonium molybdate, 3 ml. 0·0033 M-glucose-1-phosphate. pH of mixture 3·32. Temp. 70°. Total acid-labile P: 295 μg./10 ml.)

Time (min.)	P (μg./10 ml.)	Hydrolysis (%)	$k_{Mo} \times 10^3$
60	29·5	10·0	2·03
90	42	14·2	1·99
120	55·5	18·8	2·04
190	80	27·1	1·98
300	116	39·3	2·01
		Mean	**2·01**

Hydrolysis constants. As is to be expected the time course of the hydrolysis, in the case of compounds containing one phosphate bond, is that of a first-order reaction, in presence as well as in absence of molybdate. An example is given in Table 1.

The hydrolysis of ATP, a compound with two acid-labile phosphate bonds, is more complicated. The time curve (Fig. 13) of the molybdate-catalysed hydrolysis shows a break after the hydrolysis of only 50 % of the acid-labile P. The hydrolysis constants for the two phosphate groups, k_1 and k_2, were calculated from the data by making an estimate of several possible values for k_2 from that part of the curve lying between 50 and 100 % hydrolysis, and selecting that value which gave the best straight line when the hydrolysis of the terminal phosphate group, corrected for the simultaneous hydrolysis of the intermediate phosphate group at the assumed rate, was plotted on a logarithmic scale. For the experiment of Fig. 13 the hydrolysis constants of the terminal and intermediate phosphate groups in presence of molybdate, k_{Mo_1} and k_{Mo_2}, respectively, were found to be 0·078 and 0·0080. In Fig. 13 the

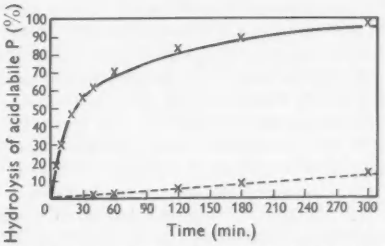

Fig. 13. Time curve of hydrolysis of the labile phosphate groups of ATP in presence and absence of ammonium molybdate (1 %). ATP concn. 0·4 mM. Temp. 70°.

curve is the calculated one, formed by the superimposition of the two first-order reactions, whereas the crosses represent the actual observations. The close agreement between observation and calculation shows that the estimates for k_{Mo_1} and k_{Mo_2} were substantially correct. The catalytic effect of molybdate on the terminal phosphate group is thus ten times greater than that on the intermediate phosphate group. In contrast to this, as has been shown

Table 2. *Effect of* 1 % *ammonium molybdate on the hydrolysis of some phosphate bonds*

(k_{Mo}: hydrolysis constant in presence of molybdate; k_c: hydrolysis constant in absence of molybdate (control).)

Substrate	pH	Temp. (°)	$k_{Mo} \times 10^3$	$k_c \times 10^3$	k_{Mo}/k_c
Acetyl phosphate	4·0	0	226	1·12	202
ATP (terminal group)	4·1	70	78	0·50*	156
Creatine phosphate	2·5	0	54·3	0·40	135
Glucose-1-phosphate	3·3	70	2·01	0·312	6·4
Fructose-6-phosphate	5·8	80	4·17	0·82	5·1
Pyrophosphate	2·2	80	24·5	5·3	4·6
Phospho-enol-pyruvate	4·0	80	55	12·5	4·4
α-Glycerophosphate	6·3	80	0·364	0·150	2·4
Glucose-6-phosphate	6·3	80	0·306	0·155	2·0

* In absence of molybdate both acid-labile phosphate groups of ATP are assumed to be hydrolysed equally (cf. text p. 290).

by Lohmann (1932), the two phosphate groups are hydrolysed at the same rate in absence of molybdate. The hydrolysis constant of the control has therefore been calculated on the assumption that the hydrolysed phosphate groups were derived equally from the terminal and the intermediate phosphate groups of ATP.

In Table 2 a number of hydrolysis constants in presence (k_{Mo}) and in absence (k_c) of molybdate are listed. The experiments were usually performed at the pH optimum of the molybdate effect and at a temperature convenient for the measurement of the reaction rate within a period of several hours.

The catalytic effect of molybdate has been expressed numerically by the ratio of k_{Mo}/k_c. No compound has yet been found where the molybdate effect was completely absent, but there is a large variation of the ratios with different compounds, ranging from about 2 to about 200.

Effects of tungstate and vanadate. Since the catalytic effect of molybdate is presumably connected with its tendency to form a heteropoly acid complex with phosphoric acid or its derivatives the question arose whether tungstate and vanadate, which form similar complexes, have also similar catalytic properties. Both tungstate and vanadate interfered with the direct colorimetric estimation of phosphate and it was therefore necessary to carry out a preliminary precipitation and separation of inorganic phosphate as magnesium ammonium phosphate ($MgNH_4PO_4$). The technique was tested on standard solutions of phosphate in presence of tungstate or vanadate and a satisfactory recovery was obtained.

Table 3. *Hydrolysis of ATP in presence of molybdate, tungstate and vanadate*

(Concn. of ATP 1686 μg. acid-labile P/10 ml. Incubated at 70° for 30 min.)

Addition	pH	Hydrolysis: P (μg./10 ml.)	Hydrolysis: Acid-labile P (%)
Nil	3·98	46	2·7
Sodium tungstate (1%)	4·32	46	2·7
Ammonium vanadate (0·9%)	4·42	76	4·5
Ammonium molybdate (1%)	4·21	650	38·6

In the experiment in which the catalytic activity was examined a third sample was set up with molybdate in which inorganic phosphate was precipitated with magnesia mixture in the same way. The result (Table 3), while showing the catalytic effect of molybdate, gave no significant indication of a similar effect of either tungstate or vanadate.

DISCUSSION

The relation of the catalytic effect to the properties of the phosphate bond. The k_{Mo}/k_c ratios of the substances investigated fall into two sharply separated groups. Acetyl phosphate, ATP-terminal phosphate and creatine phosphate show ratios 20 to 100 times higher than the rest, while the differences of ratios are comparatively small within the two groups. It is clear that there is no strict correlation between the stability of a phosphate bond towards acid and the catalytic effect of molybdate. The best example for this lack of correlation is the difference of the molybdate effect on the two acid-labile phosphate groups of ATP. Both these groups have the same heat of hydrolysis (Meyerhof & Lohmann, 1932), and are hydrolysed by hydrogen ions at the same rate (Lohmann, 1932). Yet biologically they are not equivalent since, according to current views, only the terminal phosphate group of ATP is directly utilized in transphosphorylation or hydrolysed by the specific adenosinetriphosphatases of muscle and other tissues. This differential behaviour towards enzymes is reflected in the difference in the effect of molybdate. The high-energy phosphate bonds of acetyl phosphate and creatine phosphate have a molybdate-lability similar in magnitude to that of the terminal group of ATP. On the other hand, the phosphate bond in pyrophosphate, whose energy content is high but biologically unavailable, has a molybdate lability of the same order as the intermediate phosphate group of ATP, and as most of the low-energy phosphoric esters investigated. Phospho-enol-pyruvic acid seems to be exceptional, since it combines a low molybdate lability with a phosphate bond which is both rich in energy and biologically available. But, as has recently been pointed out by Oesper (1950), the phosphate bond of phospho-enol-pyruvic acid belongs to the family of low-energy esters, and the energy released on hydrolysis is probably largely derived from the conversion of the unstable enol to the stable keto configuration.

The difference between the effect of molybdate on acid-resistant phosphate bonds, as in glucose-6-phosphate, and on acid-labile bonds of the low-energy type, as in glucose-1-phosphate, is small compared with the difference between groups of similar stability towards acid, but a different content of biologically available energy.

Mechanism of the catalytic effect and its biological significance. It has been suggested (Lipmann & Tuttle, 1944) that the catalytic effect of molybdate is due to the removal of inorganic phosphate in complex form. This view cannot be correct, for the following reasons: (1) The theory presupposes the existence of an equilibrium in which the removal of one reaction partner simply reveals, but does not accelerate, the rate of the forward reaction. The normal dynamic equilibrium would be maintained by the reverse (synthetic) reaction and molybdate would act by suppressing the latter. The theory thus leads to the assumption of the highest rate of spontaneous synthesis in exactly those phosphate

compounds, e.g. creatine phosphate and acetyl phosphate, which have the highest degree of instability. In fact, the thermodynamic equilibrium lies so far in favour of the split products that the rate of the reverse reaction must be quite insignificant. (2) The complex between molybdate and inorganic phosphate is very stable, and a small excess of molybdate is sufficient to convert inorganic phosphate quantitatively into the complex. The sustained increase of the catalytic effect with increasing molybdate concentrations, however, points to the existence of an unstable complex, the concentration of which determines the rate of the reaction. (3) The existence of a sharp pH optimum in some cases, and the fact that it varies from compound to compound, are incompatible with a mechanism which would be generally identical. It rather suggests that the rate of reaction depends on the formation of specific complexes with either a stability maximum or a decomposition maximum in a narrow range of the pH scale, depending on whether the formation or the decomposition of the complex is the rate-determining step.

It may therefore be suggested that the effect of molybdate is due to the formation of a complex with the substrate as a first step in the breaking of the phosphate bond. Complexes of molybdate with organic compounds, phosphorylated or not, are well known; those with α-hydroxy acids have been particularly studied owing to the analytical interest attached to the large increase in optical rotation which they frequently show (see, for example, Auerbach & Krüger, 1923; Meyerhof & Schulz, 1938; Krebs & Eggleston, 1943).

An evaluation of the thermal data (Fig. 2) is consistent with the assumption of an intermediary complex between catalyst and substrate. The following figures have been calculated for the overall reaction from the equation $k = Ae^{-\Delta E/RT}$, where k is the velocity constant, A the temperature-independent factor, ΔE the activation energy, R the gas constant and T the absolute temperature. A temperature-independent factor (A) of 10^{13} is obtained for the uncatalysed hydrolysis of sodium pyrophosphate, which is of the order found for 'normal' reactions. In presence of molybdate this factor is decreased to 10^7 in the case of sodium pyrophosphate and to 10^6 in the case of the terminal phosphate group of ATP, indicating a higher degree of restraint in the transition state, such as would be expected if an intermediary complex were formed. For the overall activation energy of the uncatalysed hydrolysis of sodium pyrophosphate a value of about 29,000 cal. has been calculated, which is also of the order commonly found for reactions of this type. In presence of molybdate the activation energy is lowered to about 17,200 cal. in the case of sodium pyrophosphate and to about 13,000 cal. in the case of the terminal phosphate group of ATP. If it is assumed that the catalysed hydrolysis of pyrophosphate takes place side by side with the uncatalysed reaction, the activation energy for the catalysed reaction would be about 14,000 cal. These values are of the same order as those frequently found for enzymic catalysis, which is generally assumed to depend on the formation of intermediary complexes.

The formation of a molybdate-substrate complex may perhaps explain the specific inhibition of 'acid' phosphatases by molybdate (Munemura, 1933; Courtois & Bossard, 1944; Bossard, 1947). A direct reaction of molybdate with the enzyme can of course not be excluded.

There are numerous examples of inorganic catalysts whose action is the prototype of an enzyme action, and frequently the enzyme concerned contains the same element as part of its active centre. But there is at present no reason to attach to molybdate any such significance as a phosphatase model. It is true that molybdenum is an essential trace element, at least for plants (Arnon & Stout, 1939; Arnon, 1940), but its function seems to be connected with processes of nitrogen reduction (Mulder, 1948) and nitrogen fixation (Burk & Burris, 1941; Burris & Wilson, 1945) rather than with phosphatase activity. The toxicity of molybdate, on the other hand, may be connected with its inhibitory effect on phosphatases, since Comar, Singer & Davis (1949) found disturbances of bone development in growing rats subjected to molybdate poisoning.

Passing reference should be made to some related phenomena. Lohmann (1931) was the first to point out the enhanced instability of calcium pyrophosphate and of calcium ATP compared with the sodium salts, especially in weakly alkaline solution. Lipmann & Tuttle (1944) found the same to be true for acetyl phosphate, and Bielschowsky (1950) has recently reported a special instability of the terminal group of magnesium-ATP. Bamann & Meisenheimer (1938) and Bamann & Nowotny (1948), in a series of papers, showed that the salts of polyphosphates and glycerophosphates with lanthanum and other rare earths have an increased tendency towards hydrolysis. The lanthanum effect has a well marked pH optimum of about 8, while in acid medium the hydrolysis, at least of pyrophosphate, is inhibited. It may be recalled that similar observations were made in the case of molybdate.

Analytical implications. The catalytic effect of molybdate has been used for the estimation of creatine phosphate since the work of Fiske & Subbarow (1929). The present results suggest that the same principle could be extended with advantage to distinguish between compounds of similar acid lability, but differing molybdate lability, e.g. between acetyl phosphate and ribose-1-phosphate or between ATP and glucose-1-phosphate.

In recent years the method of Lowry & Lopez (1946) has been widely used in biological research for phosphate estimations. Though the authors compared the hydrolysis of creatine phosphate, acetyl phosphate and ribose-1-phosphate under the conditions of their method and of the method of Fiske & Subbarow (1925) they did not investigate the activity-pH curve of the molybdate effect systematically, and one is left with the impression that the effect increases with increasing acidity. In the particular case of creatine phosphate it may be an advantage to carry out the analysis at pH 4, but it must not be assumed that this is true generally. For example, the pH optimum of the molybdate effect with ATP is 4, precisely the pH chosen by Lowry & Lopez; although the hydrolysis of ATP may not be significant at room temperature and at the low concentration of ammonium molybdate used in their method, it should be borne in mind that there is no particular region of the pH scale which could be judged universally safe as far as the catalytic effect of molybdate is concerned.

SUMMARY

1. The catalytic effect of molybdate on the hydrolysis of a number of phosphate compounds has been investigated.

2. The effect increases with increasing concentration of molybdate, though not in a strictly linear fashion, and also exponentially with temperature. Evaluation of the thermal data indicates a lowering of the activation energy and of the temperature-independent factor by molybdate.

3. The molybdate effect may have a well pronounced pH optimum which varies from compound to compound. In two compounds hydrolysis was inhibited by molybdate at the acid end of the pH scale.

4. For the substances investigated, the ratios of the hydrolysis constants in presence and in absence of molybdate fall into two groups. The first group consists of acetyl phosphate, creatine phosphate and the terminal phosphate group of adenosinetriphosphate, which show ratios 20 to 100 times higher than those of the second group, which comprises the other substances studied. The differences of the ratios within these two groups are relatively small. A catalytic effect of molybdate was demonstrated in all the compounds tested.

5. Although the terminal and intermediate phosphate groups of adenosinetriphosphate are hydrolysed at the same rate by acid, their hydrolysis constants in the presence of molybdate differ by a factor of 10.

6. No catalytic activity was found when molybdate was replaced by tungstate or vanadate.

7. It is suggested that the catalytic effect of molybdate involves the formation of an unstable transition complex between catalyst and substrate. The biological and analytical implications of the effect are discussed.

We are greatly indebted to Dr J. Stern for the thermodynamic calculations and discussion.

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Automatic Fraction Collectors and a Conductivity Recorder

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AUTOMATIC FRACTION COLLECTORS

In recent years a variety of designs of automatic fraction collectors have appeared (Brimley & Snow, 1949; Cuckow, Harris & Speed, 1949; Edman, 1948; Phillips, 1949; Stein & Moore, 1948).

The collectors described here possess the advantages that they are simple and cheap to construct and have given reliable service over long periods. The three machines all operate on the same principle, involving a siphon set on a balance arm which, in filling and emptying, operates a mechanical escapement. The siphon and its delivery arm are caused to rotate by means either of a spring, a twisted cord or a small weight.

The first two single-row machines differ only in arrangement of siphon, the second being the more robust. The third machine has several rows of tubes and is consequently more compact. The movement from one row to the next is accomplished with the aid of a second escapement.

Fraction collector Model 1

The apparatus described here can be readily constructed in any laboratory workshop. It consists of a circular rack of 114 test tubes opposite each of which a brass pin projects radially inwards. The siphon is suspended, by a twisted polyvinylchloride cord, from a balance beam furnished with adjustable counterweights, over the centre of the rack. The siphon empties into the test tubes through a long arm which carries a loop with overlapping ends made from glass rod (Fig. 2). The ends of the loop and the radial pins constitute an escapement which operates as follows. When the siphon is empty the radial pin presses against the upward-pointing end of the loop, and when it has become about half full the beam tilts and the loop descends, the pin passing between its ends. The arm, now free, rotates under the torque of the twisted suspending cord, and comes to rest with the downward-pointing end of the loop touching the next radial pin. When the siphon empties into the corresponding tube, the beam tilts back and the upward-pointing end of the loop comes to rest against this pin as in its starting position, the delivery arm having moved forward a distance equal to that separating the two ends of the loop. This distance, however, is small so that liquid continues to empty into the same tube in spite of the movement.

The last pin is long enough to project beyond the back of the loop and prevents it from moving further. A piece of glass tube may be slipped over any pin to act as a stop, the test tube in the corresponding position being replaced by a funnel leading to a receiver beside the rack.

Details of construction. The rack is constructed from a 30 in. square of 0·25 in. 'Tufnol' sheet (Tufnol Ltd., Perry Barr, Birmingham), to take $\frac{5}{8}$ in. test tubes at 3° intervals. The support for the beam prevents six positions from being used. The balance beam is pivoted on a pin through a slit in an upright rod set 5 in. from the centre of the rack, and carries on it two sliding weights for coarse and fine adjustments. Fixed to the rod is a cross-piece with two screws to limit the excursion of the beam. It also carries a Perspex block with two mercury pools into which a staple on the beam dips, to record the operation of the siphon when conductivity (see below) or some other property of an effluent is simultaneously being recorded.

The siphon, delivery arm and escapement loop are constructed wholly of glass. The adjustable siphon illustrated in Fig. 3 contains a hollow plunger, loosely fitting the outer tube, retained in any desired position by a wire ring. The bore of the centre tube of the plunger is equal to that of the siphon tube, so that the delivery volume is unaffected by the surface tension or density of the liquid. Tubes of 2 mm. diameter are suitable for flow rates under 30 ml./hr. For higher flow rates or relatively viscous liquids larger-bore tubes should be used. A vent pipe is provided between the siphon and the delivery arm. Because of the length of the latter it is impossible without the vent to prevent air and liquid siphoning over continuously. A small amount of liquid remains in the delivery arm, but this is reasonably constant from cycle to cycle. A tail attached to the receiver hangs vertically down below the point of support and fits loosely into a bearing just above the base board, to steady the siphon as the delivery arm fills with liquid.

Setting up apparatus. The pivot holes should be along the axis of the beam and the siphon and balance weights be symmetrical above and below so that with the siphon half full the centre of gravity of the beam is at the central pivot. The counterweight of the siphon is adjusted until the tail,

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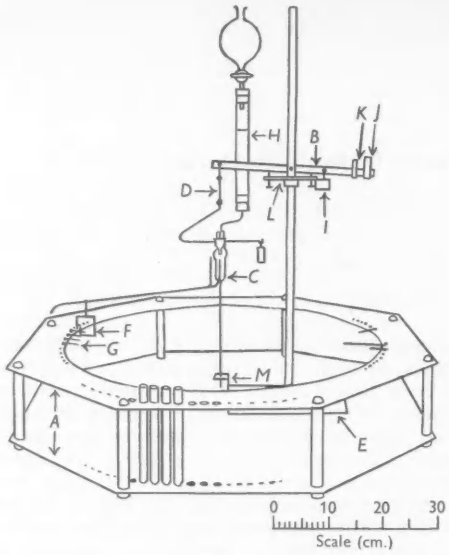

Fig. 1.

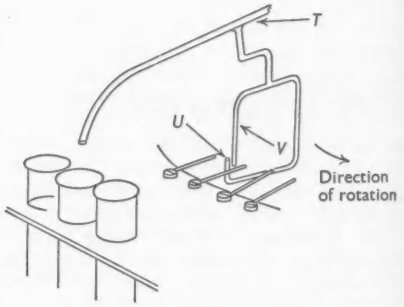

Fig. 2.

Fig. 1. Automatic fraction collector Model 1 (isometric view). *A*, rack for 114 test tubes; *B*, counter-balanced beam; *C*, adjustable siphon; *D*, twisted cord; *E*, base plate; *F*, escapement loop; *G*, escapement pins; *H*, chromatograph tube; *I*, mercury cup switch; *J*, *K*, counter-balancing weights; *L*, adjusting screws to limit beam movement; *M*, tail bearing.

Fig. 2. Detail of escapement mechanism of Model 1. *T*, delivery arm; *U*, upward-pointing end of loop; *V*, downward-pointing end of loop.

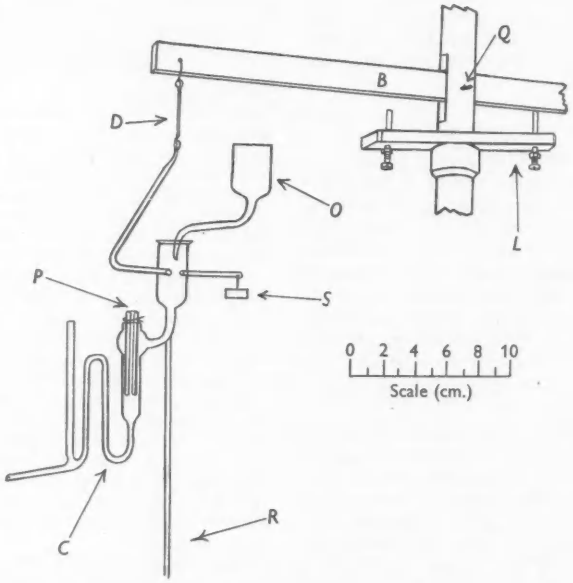

Fig. 3. Detail of beam and siphon assembly of Model 1. *B*, counter-balanced beam; *C*, adjustable siphon; *D*, twisted polyvinylchloride cord; *L*, plate carrying adjusting screws to limit beam movement; *O*, collecting cup; *P*, adjustable iner tube of siphon; *Q*, beam pivot; *R*, tail; *S*, siphon-counter-balancing weight.

removed from its bearing, is vertical. The escapement loop is adjusted until the delivery arm is directly over the tubes at both positions of the escapement. The suspension cord is given two or three turns and the tail replaced in its bearing. The balance limit screws are adjusted to give an excursion of the siphon of 5 mm. Finally, the counter-balancing weights are adjusted so that the siphon moves when half to two-thirds full. The minimum volume for satisfactory operation is about 2 ml.

This apparatus is in the Research Laboratories of Boots Pure Drug Co. Ltd., Nottingham.

a link surrounding the central pivot. This link carries two screws to adjust the excursion of the beam. The torque to rotate the beam is provided by a helical spring of about ten turns of Chromel wire around the central pivot attached at one end to the block and at the other to an adjustable collar. The siphon is connected to the delivery arm with a collar of heavy rubber tubing.

Setting up apparatus. For satisfactory operation the centre of gravity of the beam with the siphon one-half to two-thirds full must lie at the point of the pivot. Adjustment of the centre of gravity vertically and along the beam can be

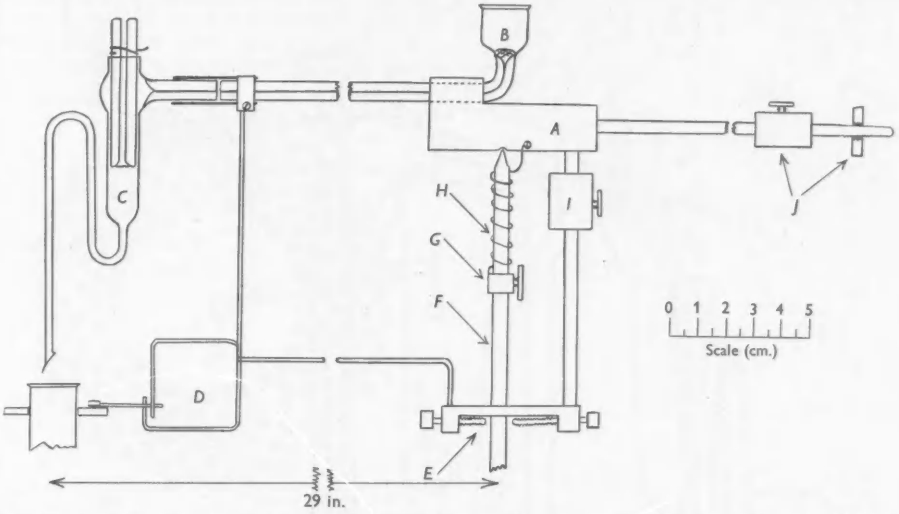

Fig. 4. Side elevation of fraction collector Model 2. *A*, brass bearing block; *B*, collecting cup; *C*, adjustable siphon; *D*, wire escapement loop; *E*, excursion-limiting screws; *F*, central pivot; *G*, spring adjustment; *H*, spring; *I*, vertical sliding weight; *J*, coarse- and fine-adjustment horizontal sliding weights.

Fraction collector Model 2

Perhaps the chief drawbacks of the model described above are the fragility of the siphon assembly and the limitation of sensitivity imposed by friction at the balance pivot. By placing the siphon at the end of the delivery arm and utilizing this as the beam, the apparatus could be made more robust and the whole could be supported on a single point, thus considerably increasing the sensitivity. The siphon delivering directly into the test tubes empties more cleanly.

Details of construction. The rack is made from 0·25 in. aluminium sheet with 120 $\frac{5}{8}$ in. holes arranged at 3° intervals. Fig. 4 shows the arrangement of the siphon assembly. A brass block has two arms at right angles carrying adjustable weights. The point of the central steel pivot bears in a depression made by a centre punch in the under side of the brass block. A delivery arm, of thick-walled 1 mm. glass capillary tube with a collecting cup at the centre, is fixed in the block with sealing wax. The escapement is fixed both to the delivery arm, and to the vertical rod projecting from the block through

effected by moving the weights; but at right angles to the beam it must be correct before leaving the workshop, otherwise the friction of the link against the pivot is excessive. The spring is then given several turns and adjusted until no vertical force is applied to the block. This spring could well be replaced by a thread and weight as in Model 3.

A small plug of cotton wool is pushed into the bottom of the collecting cup to prevent ingress of air bubbles. The minimum volume this apparatus will handle satisfactorily is about 0·5 ml.

This apparatus is in the Biochemistry Department, The Lister Institute of Preventive Medicine, Chelsea Bridge Road, London, S.W. 1.

Fraction collector Model 3

In this model (Fig. 5) the size of the rack is diminished by the use of several concentric circles of tubes. To reduce friction at the link on the central pivot, the main beam rocks on two points, whose bearings are attached to a glass tube itself pivoted on the central support. This glass tube also carries two arms, one hollow containing the weight and cord

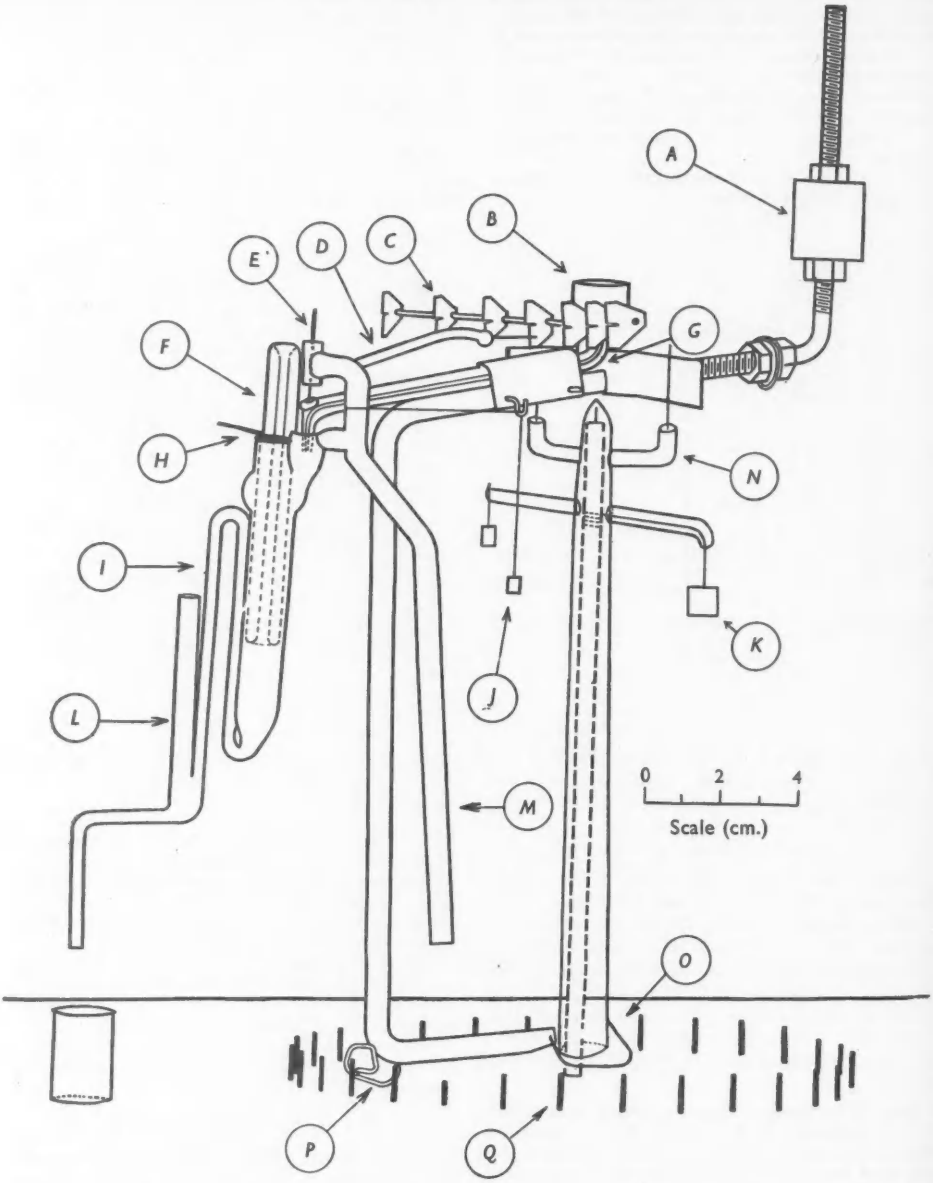

Fig. 5. Side elevation of fraction collector Model 3. *A*, centre of gravity adjustment weight; *B*, collecting cup; *C*, fins of siphon escapement; *D*, siphon arm; *E*, siphon pivot; *F*, adjustable siphon plunger; *G*, delivery arm; *H*, siphon plunger retaining wire; *I*, adjustable siphon; *J*, weight and cord to operate siphon escapement; *K*, weight and cord to operate beam escapement; *L*, siphon vent tube; *M*, siphon counter-balancing arm; *N*, main beam pivot; *O*, beam link; *P*, main escapement; *Q*, main escapement pins.

responsible for the torque necessary for the beam movement and the other supporting a counterweight which ensures that the glass tube hangs symmetrically on the central pivot. The link is replaced by a C-shaped piece of wire attached to the base of the beam to limit the excursion of the beam. In this way rubbing contact of the link and the central pivot is eliminated. The beam and delivery arm are similar to those of Model 2 except that the siphon, now pivoted at the end of the delivery arm, by the operation of an escapement on the beam once per revolution of the beam, moves outwards from one circle of holes to the next. It is a convenient geometrical fact that equal numbers of circles in con-

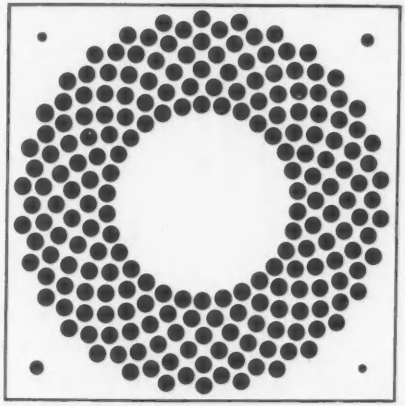

Fig. 6. Plan of closely packed arrangement of holes in test tube rack of Model 3.

centric rings packed as closely as possible lie also on arcs of circles whose centres lie on the circumference of the innermost ring and whose radius is equal to that of the innermost ring (Fig. 6). If, therefore, at the end of a pivoted beam of length equal to the radius of the innermost ring and with an escapement allowing delivery of liquid into each tube of the innermost ring, is fitted a pivoted siphon provided with a similar escapement and of the same length as the beam, delivery into each tube of successive rings is possible. The angle between successive positions of both escapements is the same. The beam escapement consists of a loop of wire (Fig. 7) as in the previous models except that the ends of the loop are nearly horizontal (in fact normal to the line joining the loop with the pivot on which the beam turns) instead of vertical as before. The loop contains also a horizontal portion at right angles to the ends of the loop. These engage with vertical pins in the rack as in the previous models, but instead of the upward excursion of the beam being limited by the link surrounding the central pivot, the horizontal portion of the loop rests against the escapement pins except in the case of a single short pin. In this case, the only difference is that the beam moves up further than usual, and its movement is finally limited by the link against the central pivot. The purpose of this larger excursion of the beam is to operate the siphon escapement which is mounted on the beam itself.

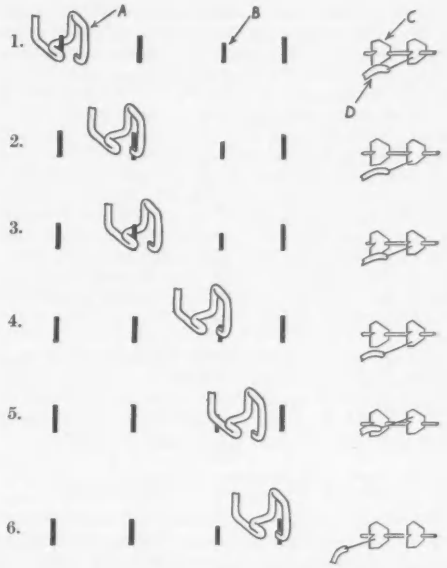

Fig. 7. Diagram of main beam escapement and siphon escapement in successive positions. *A*, main escapement; *B*, vertical pins; *C*, pins of siphon escapement; *D*, siphon arm. 1, siphon has emptied, beam tilted up. Siphon arm on lower part of fin. 2, siphon has partly filled, beam has fallen, escapement has disengaged and beam has rotated to next pin. 3, siphon has emptied, escapement still engaged. 4, siphon has filled, beam has fallen, escapement is now engaged by short pin. 5, siphon has emptied and short pin has passed under rear of escapement. Siphon arm has now passed to upper part of fin. 6, siphon has filled, beam has rotated on to next pin and the siphon arm has slipped through the slit in the fin and has rotated until arrested by the next fin. The siphon delivery arm is now over the next ring of tubes.

This escapement is in principle similar to the others, but in this case the pin is the moving member and is attached to an arm at right angles to the plane of symmetry of the siphon. The loops, seven in number, made from sheet-metal fins split horizontally with the upper half displaced sideways enough to admit the pin on the siphon arm, are mounted on the beam on an arc whose centre lies at the siphon pivot.

During the normal operation of the beam escapement the pin on the siphon arm rests against the

lower half of a fin. But when the beam escapement reaches the short pin, the beam rises more than usual and the siphon pin moves from the bottom to the top half of the fin. As the siphon fills it drops and the siphon pin passes between the leaves of the fin and the siphon delivery tube moves over to a new ring of tubes as the beam moves on to the next position (Fig. 7).

Details of construction. The rack consists of a 15 in. square of 0·25 in. 'Tufnol' sheet drilled out to take 210 16 mm. test tubes; the radius of the inner circle of thirty tubes is 3·7 in. The ring of vertical pins (height $\frac{3}{8}$ in.) has a radius of 3 in., the short pin being 0·25 in. high. The beam is constructed of 0·25 in. aluminium rod supported on a vertical pivot 0·25 in. diameter and 7·5 in. high. The delivery arm is attached to the beam by a sheet-metal staple held in position with two pins, one of which is formed into a hook to support the thread and weight coming from the siphon arm. The siphon escapement is also attached to the staple. The horizontal and vertical portions of the beam are threaded, the former to allow adjustment of the weights acting as the fine adjustment for balancing the beam. The vertical threaded portion provides adjustment of the counter-balancing weights by means of which the centre of gravity can be brought to the central pivot.

Setting up apparatus. Similar adjustments to those described for Model 2 are necessary. The smallest possible weights should be used to provide the torque necessary for the beam and siphon arm rotations. The minimum volume this instrument will handle satisfactorily is about 1 ml.

A CONDUCTIVITY RECORDER

The change in electrical conductivity in the effluent from a column provides in many cases a convenient method for following its operation. This technique is, however, only applicable where the effluent is aqueous. When the stationary phase is aqueous the

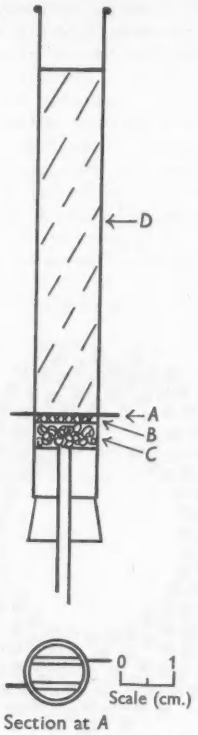

Fig. 8. Diagram of chromatogram tube fitted for conductivity measurement. *A*, platinum electrodes; *B*, cloth disk; *C*, glass wool; *D*, chromatographic filling.

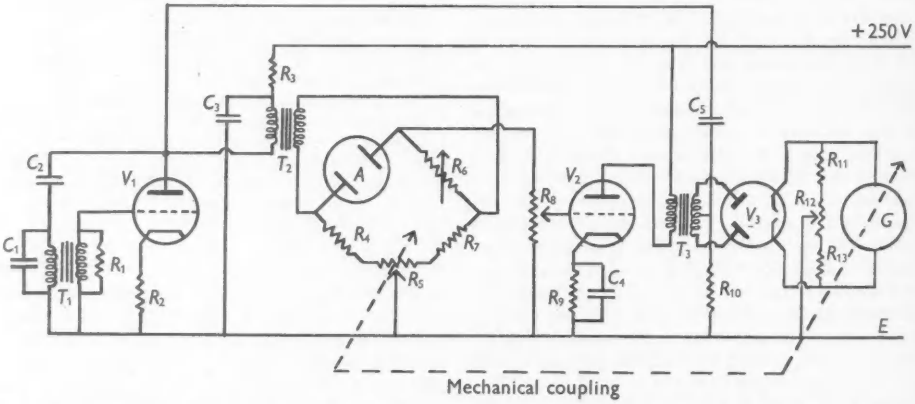

Fig. 9. Circuit diagram of conductivity recorder. Component values: condensers C_1, C_2, 0·01 μF.; C_3, 8 μF.; C_4, 50 μF.; C_5, 0·1 μF.; resistances, R_1, R_6, 20 KΩ; R_2, 500 Ω; R_3, 100 KΩ; R_5, 120 Ω; R_8, 0·5 MΩ; R_9, R_{12}, 1 KΩ; R_{10}, R_{11}, R_{13}, 10 KΩ; transformers, T_1, T_2, 3:1 ratio; T_3, 1:2 ratio, centre-tapped; valves, V_1, ML4; V_2, MH4; V_3, 2D4B. R_4 and R_7 are multiplying resistances; the scale is multiplied one, two, five and ten times with values of respectively 0, 60, 240 and 540 Ω. *A*, conductivity cell. *G*, galvanometer, mechanically coupled to R_5 in recording apparatus.

conductivity of the column itself can conveniently be measured between two horizontal electrodes of flattened platinum wire sealed into the column within 1 cm. of the bottom (Fig. 8).

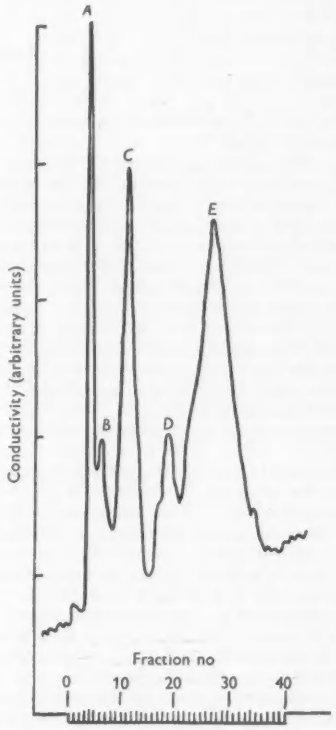

Fig. 10. Fractionation of a penicillin mixture. Tracing from conductivity-recorder chart. *A*, non-penicillin acids; *B*, *n*-heptyl-; *C*, *n*-amyl-; *D*, *n*-pent-2-enyl-; *E*, benzylpenicillin.

A commercial recording potentiometer in which the galvanometer and slide wire were mechanically coupled was used in the circuit shown in Fig. 9 to provide an automatically balanced bridge to record changes in conductivity of the column. The valve oscillator provides 1000 cyc./sec. at 0·5–1·0 V. to the bridge, whose output is amplified and fed to a phase-distinguishing circuit consisting of a bridge with two matched diodes. The d.c. output from this second bridge is fed to the recording galvanometer which automatically corrects the adjustment of the main bridge.

An additional pen is fitted to the recorder whereby the excursions of the beam of the fraction collector are marked on the same chart. The application of this technique to the analysis of a mixture of penicillins is described below.

Example of use of conductivity recorder

'Hyflo-Supercel' kieselguhr (5·0 g.) (Johns-Manville Ltd., London, S.W. 1) was thoroughly mixed with 2·5 ml. of pH 5·5 sodium citrate buffer (0·1 M), which had previously been equilibrated with a 1:1 (v/v) mixture of ethyl ether and di-*iso*propyl ether. It was made into a thick slurry with the mixed ethers and then packed into a 1 cm. diameter chromatogram tube, 15 cm. long, fitted with platinum electrodes. The tube was set up in the automatic collector with a 3·75 ml. receiver and the electrodes were connected to the conductivity recorder. The mixed ether solvent was run through the column from a separating funnel until the conductivity was steady. Then, without interrupting the flow of solvent, a solution of 10 mg. of crude penicillin acids (1000 i.u./mg.) in 1 ml. of the mixed ethers was carefully run on to the surface of the kieselguhr from a long capillary pipette inserted down through the separating funnel.

Fig. 10 is a photographic reproduction of the record obtained. Forty fractions were collected, non-penicillin acids being present in fractions 3–5, *n*-heptylpenicillin in 6–8, *n*-amylpenicillin in 9–14, *n*-pent-2-enylpenicillin in 15–20 and benzylpenicillin in 21–35.

SUMMARY

1. Three types of automatic fraction collector working on essentially the same principles are described, all of which can be easily constructed in any laboratory workshop.

2. A conductivity recorder is described that provides automatic registration of the changes in electrical conductivity of the stationary aqueous phase of a partition chromatogram together with the volume of effluent passing through the column.

3. An example of the application of an automatic fraction collector and the conductivity recorder to the analysis of a mixture of penicillins is described.

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Some Aspects of Phosphorus Metabolism in Bone Marrow

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The chemical composition and the metabolism of bone marrow have been recently studied by several investigators, including Warren (1934, 1940); Schultze (1941); Goldinger, Lipton & Guzman Barron (1947); Needham, Cohen & Barrett (1947); Davidson, Leslie & White (1947, 1948, 1950); Evans & Bird (1949); Dietz (1949) and Dietz & Steinberg (1949).

This paper deals with certain aspects of phosphorus metabolism in bone marrow, and summarizes results obtained in the course of the last 4 years (Lutwak-Mann, 1947*a*, *b*, 1949, 1951; Lutwak-Mann & Gunz, 1949), based on experimental material from some 60 rabbits and well over 1500 rats. It was necessary to use relatively large numbers of animals because of the variations observed in the phosphorus content of the bone marrow of normal animals. These were due to species differences as well as, within one species, to not inconsiderable fluctuations between members of different breeds. To reduce the influence of these and other factors, such as age and sex, young adults only were used, the rabbits being litter mates of the same breed, and the rats males of a highly inbred laboratory stock.

The phosphorus content of rabbit and rat bone marrow was first investigated in normal untreated animals; next, the effect was examined upon the phosphorus content of extraneous factors such as changes in the composition of food leading to undernourishment or vitamin deficiency; furthermore, phosphorus metabolism was followed in the bone marrow of animals exposed to the action of myelotoxic agents such as mustard gas and X-rays. In addition, *in vitro* experiments were carried out to study the phosphorus distribution under conditions of tissue autolysis.

MATERIAL AND METHODS

Material. The bone marrow was removed from the long bones (femur, tibia, humerus), after dissection of the epiphyses, by means of compressed air. The quantities of material collected in this manner varied in rabbits between 3 and 11 g. and in rats from 120 to 200 mg. fresh weight per animal. The animals were thoroughly bled after decapitation and little blood was present in the normal bone marrow; on several occasions the haemoglobin content of the marrow samples was estimated and found to correspond to blood diluted 1 in 15 to 1 in 20; it is not likely, therefore, that the residual blood could have affected the analytical results to any appreciable degree.

Methods. Phosphorus was determined by means of the Fiske & Subbarow (1925) method. In the text below 'directly determinable P' applies to phosphate which reacted directly with molybdate, 'readily hydrolysable P' is the fraction which hydrolysed at 100° in N-HCl in 10 min. ($P_{10} - P_0$), and 'difficultly hydrolysable P' corresponds to the difference between total acid-soluble P and the fraction which hydrolysed in 120 min. ($P_{tot.} - P_{120}$). Adenosinetriphosphate (ATP) and NH_3-N were determined by the method of Parnas & Lutwak-Mann (1935). Total nucleic acid, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and lipid P were estimated according to the Schmidt & Thannhauser (1946) procedure. For the estimation of acid-soluble P precautions were taken to avoid the presence of bone splinters.

Mustard gas (*H*) was routinely applied to rats (180–220 g.) as follows: 0·1 ml. of 4% (v/v) solution of *H* in ethyleneglycol monoethyl ether was painted on the plucked skin (roughly 1 in. square) on the back of an animal anaesthetized with ether, left on for 5 min. and washed away with ethanol, soap and water. Under such conditions a certain amount of *H* evaporates, and it is probable that less than half the amount applied actually penetrates the rat skin. Nevertheless, when these conditions were strictly adhered to, a remarkably reproducible picture developed in response to the treatment. The animals stopped eating and drinking 24–36 hr. after the start of the experiment; they had severe gastro-intestinal disturbances from the 2nd or 3rd day onwards, and showed all the typical symptoms of systemic *H* poisoning between the 3rd and 4th day, with chromodacryorrhoea and prostration leading to coma; there was severe leucopenia at that time. The animals which survived the 4th–6th days were likely to live on for some weeks, but frequently late deaths occurred at 3–4 weeks.

Mustard sulphoxide (*HO*), which unlike *H* has unreactive chlorine atoms and is non-vesicant, was administered by subcutaneous injection. The symptoms depended upon the dose: with amounts corresponding roughly to those of the cutaneous *H* application, i.e. 0·01–0·02 mg./g., the animals were practically unaffected except for a slight loss of weight; 0·05 mg./g. produced some toxic effects, and 0·1 mg./g. was nearly as toxic as the routine dose of *H*. But even then, although the animals showed severe gastro-intestinal disturbances, they were never quite so ill as those which had been treated with *H*; in particular the nervous symptoms were much less pronounced. Leucopenia of moderate to severe degree was evident after the administration of 0·05–0·1 mg./g.

Dimethyldithiocarbamate (DMT), a substance originally introduced by Cameron & Rydon (1944) as an antidote in systemic *H* poisoning, was administered subcutaneously in

doses of 0·1–0·2 mg./g.; there were no symptoms except for a passing somnolence soon after the injection. Diethyldithiocarbamate (DET), urethane and thiourea were given by subcutaneous injection in the above quantities and had, when given alone, no visible ill effects upon the rats.

A combination of small, by themselves ineffectual, doses of *HO* (0·01–0·02 mg./g.) with DMT was invariably followed by severe toxic symptoms which resembled those characteristic of either *HO* in high dosage (0·1 mg./g.) or of *H* itself. Even when DMT was injected not simultaneously with, but 7–10 hr. after, a small dose of *HO*, the clinical symptoms of *H* poisoning could still be produced.

A combination of small amounts of *HO* with thiourea also led to symptoms of some severity; the addition of urethane to a low dose of *HO* did not affect the general condition of the rats, but, judging from results obtained with nucleic acid P in the bone marrow (Table 8), there was a distinct effect upon this tissue.

Irradiation experiments were carried out with rabbits and rats; some of the results were described earlier (Lutwak-Mann & Gunz, 1949). The animals received single doses of röntgen radiation to the body as a whole; a dose of 1000 r. was given to 2·5–3·5 kg. rabbits, and 500 r. to 180–240 g. rats. The irradiations were carried out by the Staff of the Department of Radiotherapeutics, University of Cambridge, by kind permission of Prof. J. S. Mitchell, with a 220 keV. Maximar machine, fitted with an Al filter only; a dose rate of 130 r./min. was applied in a 180 sq.cm. field. Rabbits which received a dose of 1000 r. usually showed loss of weight for about 2 weeks, a few also developing intestinal symptoms; none died during the 2–3 weeks' experimental period. Rats varied in their response to irradiation; some appeared relatively slightly affected, whereas others succumbed within a few days with severe symptoms of widespread haemorrhage and diarrhoea. The surviving animals, about half the number of those exposed to radiation, showed loss of hair and greying, and a poorer increase in weight than the controls; their spermatozoa when examined at 8–10 weeks after exposure were largely immotile and disintegrated.

A small number of experiments was carried out in which bone marrow was exposed *in vitro* to large doses of X-rays (10,000 and 20,000 r., at a dose rate of 1000 r./min., and 250,000 r. in the course of 20 min.); the bone marrow was thinly spread on a flat watch glass and irradiated, while the control sample was kept for the same length of time; both samples were then used for P analysis, without further incubation.

For the nutritional experiments control rats were maintained on a full synthetic diet which consisted of 23 parts of light white casein, 40 of rice starch, 17 of cane sugar, 15 of arachis oil, 5 of salt mixture and 2·5 of cod liver oil, supplemented with 7·5 parts of dried yeast; the vitamin B-deficient rats received the same food without the yeast supplement. Vitamin B_1 deficiency was produced by feeding the following diet: cane sugar 60 parts, light white casein 20, arachis oil 15, salt mixture 5, cod liver oil 2·5, supplemented by 12 parts of autoclaved Marmite for the deficient, and the same quantity of untreated Marmite for the control animals. Vitamin B_1 used in these experiments was crystalline aneurin (Roche Products Ltd.); 'vitamin B complex', kindly supplied by Dr E. Kodicek, Nutrition Laboratory, University of Cambridge, was made up as described by Kodicek & Carpenter (1950); vitamin B_{12} was 'Cytamen' (Glaxo); folic acid was 'Folvite' (Lederle).

RESULTS

Phosphorus compounds in the bone marrow of normal rabbits and rats

The distribution of various phosphorus fractions in fresh bone marrow is represented in Table 1; it will be seen that in rabbit as well as in rat bone marrow the level of directly determinable P showed fluctuations; these are thought to be largely the outcome of enzymic activity of the bone marrow and caused by the unavoidable delay in the collection of the material. A greater amount of readily hydrolysable P was found than corresponded to ATP pyrophosphate calculated on the basis of the ATP amino N determination; this discrepancy, of varying extent, was presumably due to the presence in bone marrow of acid-labile P compounds other than ATP. The variations encountered in total acid-soluble P in the bone marrow of rabbits may be considered real, as it was found that this particular phosphorus fraction was relatively stable during incubation at room temperature (Table 3); but in rat bone marrow total acid-soluble P rose markedly during incubation (Table 4) so that the variations in its content may be at least partly due to this circumstance.

Table 1. *Distribution of phosphorus compounds in bone marrow of rabbits and rats*

(Average values calculated from fifteen determinations, except in the case of rat nucleic acid P which is based on forty determinations. P_0 = phosphorus determined as phosphate which reacts directly with ammonium molybdate; P_{10} and P_{120} = phosphorus which appears as orthophosphate after 10 and 120 min. hydrolysis with N-HCl; $P_{tot.}$ = total phosphate after incineration; ATP amino N = nitrogen of the adenine amino group of ATP determined as NH_3 after enzymic deamination; P_{lipid} = phospholipin phosphorus; $P_{nucl.}$ = total nucleic acid phosphorus.)

	Acid-soluble P (mg./100 g.)				ATP amino N (mg./100 g.)	P_{lipid} (mg./100 g.)	$P_{nucl.}$ (mg./100 g.)
	(P_0)	($P_{10} - P_0$)	($P_{tot.} - P_{120}$)	($P_{tot.}$)			
Rabbit							
Average	5·7	7·7	10·6	40	0·7	25	77
Range	3·8–8·3	3·6–11·0	8·0–12·6	32–48	0·4–1·6	27–37	48–123
Rat							
Average	35	11	28	95	—	56	250
Range	29–42	7–14	24–36	72–115	—	47–60	220–300

Lipid P showed fairly constant values in rabbit, and somewhat higher ones in rat, bone marrow. The difference was greatest in the nucleic acid P content of rabbit and rat bone marrow. In rabbits chosen at random, values for nucleic acid P differed from each other very considerably; better agreement was found in litter mates (Table 2). On the other hand, very good reproducibility existed with regard to nucleic acid P in members of the laboratory stock colony of hooded rats, where there appeared to be some minor sex difference which, however, was eliminated by the exclusive use of young adult males. The concentration of nucleic acid P in rat bone marrow was on the average three times higher than in rabbits.

Table 2. *Nucleic acid phosphorus in the bone marrow of litter mate rabbits*

Litter	Nucleic acid (mg. P/100 g.)
A	113, 123, 98, 120
B	57, 62, 60
C	72, 79
D	88, 95

Changes in the distribution of bone marrow phosphorus in the course of incubation in vitro

To study the behaviour of acid-soluble P during incubation of bone marrow, the following experiments were set up. Rabbit bone marrow from several animals was collected, with the least delay, on muslin fixed on a tared beaker; the material was gently squeezed through muslin, weighed and dispersed in 5–8 vol. of water or saline. With the aid of a blunt pipette samples were removed from the suspension, sedimentation of particles being prevented by frequent stirring and shaking.

For initial values a sample was at once deproteinized with trichloroacetic acid; others were incubated with some toluene at 24° for 4 hr., either in air or in nitrogen, and then deproteinized. From the protein-free filtrate samples were taken for the estimation of directly determinable and total P, as well as of ATP and ammonia N; the rest was brought to pH 8·3 and precipitated with barium acetate + 5 vol. of ethanol containing ammonia; after removal of barium, this fraction was used for the determination of directly determinable, readily and difficultly hydrolysable P. The results of a representative experiment of this kind are in Table 3. It will be seen that in rabbit bone marrow only a small proportion of acid-soluble P was not barium-precipitable; total acid-soluble P under these incubation conditions increased only slightly; however, there was an appreciable accumulation of inorganic P in the course of incubation, particularly in the absence of oxygen. Readily hydrolysable P increased aerobically, but was diminished in the absence of oxygen, whereas ATP amino N was well maintained in air, and even in nitrogen somewhat less than half the initial amount was still present at the end of the incubation; as in fresh bone marrow, more readily hydrolysable P was found than could be accounted for by the ATP pyrophosphate P. The decrease in the difficultly hydrolysable P fraction, due to the enzymic activity of the bone marrow, was presumably responsible for the rise in inorganic P. The ability of bone marrow to dephosphorylate various added phosphate esters was demonstrated in separate experiments and referred to earlier (Lutwak-Mann, 1949). The above processes concerned with the phosphorus metabolism of the bone marrow were accompanied by production of ammonia, irrespective of the presence of oxygen, in amounts which exceeded several times the content of preformed ATP amino N.

The behaviour of acid-soluble as well as nucleic acid P was also examined in rat bone marrow. It was found that at the end of a few hours incubation at 38° of 150–200 mg. of bone marrow in 2 ml. of water or buffer solution with some toluene, there was an appreciable increase in total acid-soluble P and a fall in nucleic acid P; estimations of DNA and RNA showed that during a 5 hr. incubation the DNA/RNA ratio increased to 3·4 from the initial value of 1·75, indicating that the breakdown of RNA was chiefly responsible for the loss in total nucleic acid content.

Table 3. *Changes in the content of acid-soluble phosphorus compounds and ammonia during aerobic and anaerobic incubation of rabbit bone marrow*

(Incubation 4 hr. at 24° in presence of toluene. Deproteinization with trichloroacetic acid. From the filtrate samples taken for determinations of P_0, $P_{tot.}$ and NH_3; in another sample ATP amino N determined by enzymic deamination; the rest of the trichloroacetic acid filtrate precipitated with barium acetate at pH 8·3 in presence of 5 vol. ethanol. For symbols see Table 1.)

	P in trichloroacetic acid filtrate (mg./100 g.)		ATP (mg./100 g.)		P fractions in Ba precipitate (mg./100 g.)				Free ammonia (mg. NH_3-N /100 g.)
	P_0	$P_{tot.}$	Estimated NH_2-N	Calculated labile P	P_0	$(P_{10} - P_0)$	$(P_{tot.} - P_{120})$	$P_{tot.}$	
Before incubation	11·2	34·0	0·76	3·37	11·5	5·0	10·6	29·3	1·30
After aerobic incubation	15·2	35·6	0·61	2·70	16·5	7·6	4·8	29·8	3·41
After anaerobic incubation	26·0	36·2	0·32	1·42	24·6	2·1	2·2	32·0	3·58

To establish how far these findings were specific for bone marrow, similar experiments were carried out with suspensions of rat spleen. The results for bone marrow and spleen are in Table 4. They show that there was a fall in the content of nucleic acid P in the spleen, but it was less marked than in the bone marrow; acid-soluble P increased in both tissues during incubation; the rather high initial level of acid-soluble P in the rat bone marrow in this particular instance was probably the result of delay in the collection of sufficient experimental material. Since phospholipin P was not determined in these experiments, it was not possible to decide what proportion of the acid-soluble P increment was due to lipid breakdown and how much was derived from nucleic acids. Considerable amounts of ammonia accumulated during the incubation of rat bone marrow, and somewhat less with rat spleen. When suspensions of bone marrow (or spleen) were heated for 10 min. at 85°, or precipitated with trichloroacetic acid, and then incubated, the processes described here were largely abolished. In rabbit bone marrow incubated at 38° there was also a loss in nucleic acid P, but to a much smaller extent than in rat bone marrow.

Several experiments were carried out to study some details of the enzymic breakdown of nucleic acid P in rat bone marrow. It was found that whereas the reaction as assessed by the disappearance of total nucleic acid P was largely independent of pH, DNA appeared to be less resistant at low than at high pH values; on the other hand, even more RNA disappeared in an alkaline than in an acid medium. The decomposition of nucleic acid proceeded readily in suspensions in water, and in phosphate, veronal and glycine buffers (Table 5). Ammonia was also determined in these experiments; somewhat more of it was produced under conditions which enhanced the breakdown of RNA, that is at higher pH values.

The *in vitro* exposure of rat bone marrow to doses of röntgen radiation ranging from 10,000 to 250,000 r. did not affect the content of nucleic acid P. The addition *in vitro* of *H*, *HO*, or *HO* together with

Table 4. *Changes in the content of acid-soluble phosphorus, nucleic acid phosphorus and ammonia during incubation of rat bone marrow and spleen*

(Suspensions of fresh bone marrow or ground spleen, 150–200 mg. tissue in 2 ml. water, incubated in presence of toluene, and deproteinized with trichloroacetic acid. Total acid-soluble P ($P_{ac.\ sol.}$) and NH_3 determined in the trichloroacetic filtrate. Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) determined as P in the trichloroacetic acid precipitate.)

	Incubation time (hr.)	P compounds (mg. P/100 g.)			DNA/RNA	NH_3 (mg. N/100 g.)
		$P_{ac.\ sol.}$	DNA	RNA		
Bone marrow	0	129	153	87	1·75	3·8
	3	210	120	45	2·66	28·2
	5	232	109	32	3·40	43·0
Spleen	0	78	100	66	1·51	3·5
	1	95	95	58	1·67	10·5
	3·5	131	87	50	1·74	16·3
	5	162	72	40	1·80	22·0

Table 5. *Effect of* pH *on the breakdown of deoxyribonucleic and ribonucleic acid and the formation of ammonia in rat bone marrow incubated* in vitro

(Rat bone marrow, 150–200 mg., suspended in 2 ml. water or 0·06 M-buffer, control deproteinized at once, the other samples incubated at 38° in the presence of toluene, and then deproteinized with trichloroacetic acid; ammonia determined in the protein-free extract, DNA and RNA in the trichloroacetic acid precipitate.)

Exp. no.	Medium	pH	Incubation time (hr.)	DNA (mg. P/100 g.)	RNA (mg. P/100 g.)	NH_3 (mg. N/100 g).
1	—	—	0	139	81	10
	Water	6·0	2	114	50	22
	Veronal-acetate	4·9	2	125	44	23
		6·9	2	136	41	26
		8·0	2	138	36	29
2	—	—	0	160	80	11
	Veronal-acetate	6·9	3·5	130	45	35
		8·0	3·5	147	40	41
	Phosphate	7·0	3·5	149	34	41
		8·0	3·5	153	33	48
3	—	—	0	165	80	—
	Glycine-HCl	1·9	1	122	67	—
	Glycine-NaOH	12·0	1	149	54	—

DMT, in a final concentration of 0·005 M to aqueous suspensions of incubated bone marrow, did not alter appreciably the rate of breakdown of nucleic acid P as compared with samples incubated without these additions.

The dependence of the nucleic acid phosphorus content in rat bone marrow upon variations in the composition of food

As previously stated, the content of nucleic acid P in the bone marrow of rats belonging to an inbred stock varied little in animals of the same age and sex. In connexion with research on the action of myelotoxic agents which frequently cause deterioration in the general condition of the animal as well as diminished food intake, it was essential to establish to what extent the bone marrow content of nucleic acid P depended upon quantitative and qualitative alterations of the stock diet.

Rats weighing 140–180 g. were maintained for 4–5 weeks on diets deficient in B group vitamins, or in vitamin B_1 only; control animals were subdivided into groups some of which were pair fed with the deficient rats, and others which were fed *ad lib.* In addition, several of the deficient as well as the non-deficient rats were treated parenterally for 2 weeks with 'vitamin B complex' or aneurin, and also with vitamin B_{12} or folic acid, during which period they were either pair fed with deficient untreated animals, or allowed to consume food without restriction. Some of the results were reported earlier (Lutwak-Mann, 1951). In addition to the bone marrow the secretory function of the male accessory glands of reproduction, which had previously been shown to be very sensitive to nutritional deficiency (Lutwak-Mann & Mann, 1950), was also examined in all these experimental animals. The findings have been summarized briefly (Lutwak-Mann & Mann, 1951).

Table 6 shows the effect of dietary changes on the nucleic acid P content of rat bone marrow. General vitamin B deficiency, or B_1 deficiency, reduced considerably the content of this phosphate fraction in the bone marrow; but a quantitative restriction of non-deficient food by pair feeding also led to a distinct decrease. As regards treatment with vitamins, in animals receiving the full diet an excess of aneurin was without effect; the condition in the deficient animals could be improved with vitamins of the B complex or with aneurin only if at the same time there was no quantitative limitation of food intake. The results in Table 6, which relate to deficient rats treated with vitamin B_{12} or folic acid, were carried out on pair-fed rats, to study the ability of these substances to stimulate rat bone marrow irrespective of food consumption. However, treatment of rats with vitamin B_{12} or folic acid, unlike B complex or aneurin, failed to improve the general health of the animals and had no beneficial effect so far as the content of nucleic acid P in the bone marrow was concerned.

The changes brought about by inadequate feeding were not irreversible: when the deficient animals

Table 6. *Effect of diet on the nucleic acid phosphorus content of rat bone marrow*

(Groups nos. 1, 5 and 10 comprise ten animals each, the rest four animals each.)

Group no.	Diet	Nucleic acid (mg. P/100 g.) DNA Mean	DNA S.D.	RNA Mean	RNA S.D.
1	Full synthetic diet	166·1	9·34	85·5	6·95
2	Full synthetic diet + 200 μg. aneurin daily	166·5	3·54	82·5	4·95
3	Full synthetic diet restricted by pair feeding with vitamin B-deficient rats	140·5	9·19	70·5	4·95
4	Full synthetic diet restricted by pair feeding + 100 μg. aneurin daily	139·0	8·94	73·5	0·71
5	Vitamin B-deficient diet	106·1	23·24	58·1	7·18
6	Vitamin B-deficient + 100 μg. aneurin daily; unrestricted food intake	140·8	12·34	97·5	9·98
7	Vitamin B-deficient + 0·4 ml. 'B-complex'; unrestricted food intake	147·0	12·73	88·5	7·78
8	Vitamin B-deficient + 10 μg. vitamin B_{12} daily; pair fed	122·7	30·00	69·3	22·28
9	Vitamin B-deficient + 0·5 mg. folic acid daily; pair fed	101·0	18·39	53·5	11·18
10	Vitamin B_1-deficient diet	85·2	20·50	52·8	14·58
11	Vitamin B_1-deficient + 100 μg. aneurin daily; unrestricted food intake	123·0	16·24	67·5	17·68

were placed on full diet for about 3 weeks a gradual recovery took place, and normal or near normal values of nucleic acid P were found in the bone marrow. It may be added here that whereas it was possible to restore completely the secretory function in the accessory glands of deficient animals, regardless of food consumption, by treatment with testosterone propionate or chorionic gonadotrophin, these hormones had no effect whatsoever upon the content of nucleic acid P in the bone marrow.

The effect of mustard gas (H) and mustard sulphoxide (HO) on the phosphorus content of bone marrow

In continuation of earlier studies which dealt mainly with systemic effects of *H* and *HO* poisoning with particular reference to the glycolysis of bone marrow and gastric mucosa (Lutwak-Mann, 1947*b*), experiments were carried out to examine the phosphorus content in the bone marrow of animals treated with these substances.

Table 7. *Effect of mustard gas (H), mustard sulphoxide (HO), and HO in combination with dimethyldithiocarbamate (DMT), on acid-soluble and nucleic acid phosphorus content of rat bone marrow*

(Rats, 180–200 g. body weight, on full synthetic diet. Injected doses are expressed in mg./g. body weight; *H* applied cutaneously, 0·1 ml. 4% solution in ethylene glycol monoethyl ether for 5 min.; *HO*, 0·02 mg., and DMT, 0·2 mg., subcutaneously. The values for total acid-soluble P ($P_{ac.\ sol.}$) and nucleic acid P ($P_{nucl.}$) are average figures from three to four rats, expressed as mg. P/100 g. bone marrow. Ranges in brackets.)

Time after injection (days)	Untreated controls		*H*		*HO*		*HO* + DMT		DMT	
	$P_{ac.\ sol.}$	$P_{nucl.}$	$P_{ac.\ sol.}$	$P_{nucl.}$	$P_{ac.\ sol.}$	$P_{nucl.}$	$P_{ac.\ sol.}$	$P_{nucl.}$	$P_{ac.\ sol.}$	$P_{nucl.}$
1	93 (72–110)	249 (238–250)	68* (58–70)	168* (148–180)	—	—	—	—	—	—
2	—	—	51* (50–55)	62* (50–71)	80 (75–100)	250 (228–260)	53* (49–68)	75* (62–80)	81 (70–97)	222 (200–241)
5	—	—	—	—	—	250 (220–258)	96† (80–118)	178† (120–188)	—	—
7	—	—	102 (98–110)	225 (178–240)	—	—	105 (90–128)	233 (180–245)	—	—
10	87 (78–115)	256 (242–262)	94 (88–97)	222 (196–250)	70 (50–108)	262 (250–268)	130‡ (125–136)	234‡ (210–247)	78 (67–88)	239 (223–244)

* Admixture of blood in bone marrow; diarrhoea.
† Bone marrow of soft consistency but no blood.
‡ Bone marrow strikingly pale and fatty.

Table 8. *Effect of various doses of mustard sulphoxide (HO), alone and in combination with diethyldithiocarbamate (DET), urethane, and thiourea, on the content of acid-soluble and nucleic acid phosphorus in rat bone marrow, 48 hr. after administration*

(Rats, 200–220 g. body weight, on stock diet. P values are averages from two to three rats; for symbols see Table 1.)

Substances injected	Dose (mg./g. body weight)	P fractions (mg. P/100 g.) Acid-soluble P $(P_{10} - P_0)$	Acid-soluble P $P_{tot.}$	Nucleic acid P DNA	Nucleic acid P RNA	DNA/RNA
HO	0·01	9·0	95	175	88	1·97
	0·05	6·3	100	106	80	1·32
	0·10	—	78*	59*	47*	1·25
HO + DET	0·01 0·10	4·3	61	46	39	1·18
DET	0·10	—	65	169	81	2·08
HO + urethane	0·01 0·10	7·1	102	144	82	1·75
HO + thiourea	0·01 0·10	—	100†	87†	62†	1·40
Urethane	0·10	10·0	81	167	83	2·01
Thiourea	0·10	—	93	159	79	2·01

* Considerable admixture of blood in bone marrow; diarrhoea. † Slight admixture o f blood in bone marrow.

Table 7 records experiments in which a comparison was made of the action upon acid-soluble and nucleic acid P in bone marrow, of roughly equivalent doses per g. body weight of *H* and *HO*, the latter being used alone and in combination with DMT, in rats which survived the treatment for 10 days. It will be seen that at 24 and 48 hr. following the cutaneous application of *H*, parallel with the development of clinical symptoms, there was a considerable fall both in acid-soluble and in nucleic acid P; a dose of 0·02 mg./g. *HO* alone was without effect, but when administered together with DMT, it provoked changes in the bone marrow analogous to those due to *H* itself. Towards the end of the 10-day experimental period the nucleic acid P values were restored to nearly the normal level; it was interesting to note a rise in acid-soluble P at this stage in the bone marrow of rats treated with *HO* + DMT. The latter by itself had little effect upon the phosphorus content of bone marrow.

Table 8 illustrates the early effects upon acid-soluble and DNA and RNA P of various doses of *HO*, and also of small doses of *HO* administered together with DET, urethane and thiourea. *HO* (0·1 mg./g.) affected the bone marrow in the same manner as the (much smaller) routine dose of *H*. DET was at least as effective as DMT, when used in combination with *HO*. Urethane alone was without effect; however, together with *HO*, it produced a slight but distinct fall in nucleic acid P; on the other hand, thiourea, ineffective alone, markedly potentiated the *HO* effect in bone marrow. The DNA/RNA ratio remained unchanged in experiments with small amounts of *HO*, or in those with urethane or thiourea alone; it fell, however, in all those instances where the combined action of these substances caused toxic symptoms in the animals and damage in the bone marrow, thus indicating that the DNA fraction suffered more under these conditions than RNA.

Effect of röntgen radiation on the phosphorus content in the bone marrow of rats and rabbits

In rats which received a single dose of radiation to the body as a whole, the nucleic acid P content of the bone marrow, and also of the spleen and testes, was examined at various times after exposure. The various aspects of these experiments are set out in Table 9 which illustrates the changes in total nucleic acid P from the 6th hour onwards after irradiation; Table 10 which provides data, based on a similar experimental series but with rather younger animals, concerning the DNA and RNA contents; Table 11 which summarizes the details of the general effects of irradiation as well as the chemical changes during a 10-week experimental period; and Fig. 1 which shows the DNA and RNA distribution in the bone marrow of rats irradiated with a single 500 r. dose of X-rays.

From the data in Table 9 it is evident that a few hours after irradiation there were gross changes in the appearance and consistency of the bone marrow owing to tissue damage which was accompanied by a fall in nucleic acid P, in some instances to about half the control value. The decline in nucleic acid content was most marked at 3–4 days, but low values persisted in most of the surviving animals for 2–3 weeks; thereafter, parallel with the gradual improvement in the general condition, and together with evidence of cellular recovery based on microscopic examination, there was a rise in the content of nucleic acid P, so that when examined at 50 days several, though not all, of the animals showed normal values for this phosphorus fraction (Tables 9

Table 9. *Effect of total body exposure to X-rays (500 r.) on total nucleic acid phosphorus in bone marrow, spleen and testes of rats*

(Averages based on values with three rats (range in brackets), except the 7-day value which is based on one rat only. Rats 200–225 g. at start of experiment.)

Time after exposure	Bone marrow (mg. P/100 g.)	Spleen		Testes	
		(mg. P/100 g.)	(mg. P/organ)	(mg. P/100 g.)	(mg. P/organ)
Untreated	264 (252–270)	179 (168–197)	1·7	66 (63–70)	0·65
6 hr.	133* (100–163)	—	—	—	—
24 hr.	123* (89–140)	—	—	—	—
4 days	78 (48–118)	82 (79–85)	0·45	71 (55–88)	0·9
7 days	82	130	0·62	79	0·8
21 days	158 (114–202)	275 (213–300)	3·6	51 (48–76)	0·5
50 days	250 (158–263)	177 (172–183)	1·2	46 (40–53)	0·65

* Bone marrow semi-fluid; admixture of blood.

and 10). The spleen underwent considerable fluctuations in weight as a result of irradiation; it was therefore of interest to present not only the concentration but also the content per organ of nucleic acid P; it was found that an early decrease was followed by a rise 3 weeks after irradiation which, however, was transient, so that more normal values were found at the later experimental stage (Table 9). In testicular tissue there were no marked early changes either in organ weight or in nucleic acid P level; at 3 weeks, however, a certain diminution in both became evident, which appeared progressive; this may well be linked with the fact that when spermatozoa were examined in irradiated surviving rats at 8–10 weeks after exposure they were almost completely immotile and largely disintegrated.

It will be noted that the DNA fraction appeared more sensitive to the damaging effect of radiation than the RNA (Table 11; Fig. 1). This applied not only to the bone marrow but also to the spleen, but was not very obvious in testicular tissue. Similar behaviour of the two nucleic acids was also observed in systemic *H* poisoning.

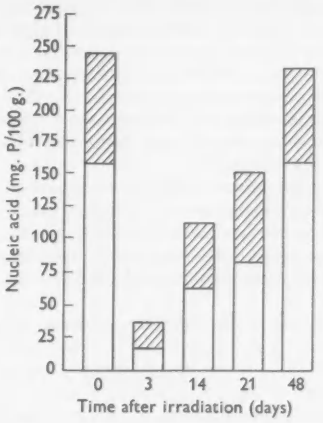

Fig. 1. Effect of total body exposure to a single dose of X-rays (500 r.) on the content of deoxyribonucleic acid (DNA) P (unshaded areas), and ribonucleic acid (RNA) P (shaded areas) in rat bone marrow.

Table 10. *Deoxyribonucleic (DNA) and ribonucleic acid (RNA) phosphorus content of rat bone marrow, spleen and testes, at various periods after whole body exposure to X-rays* (500 *r.*)

(Average values from two animals; some of the controls were examined at the beginning, the rest at the end of the experimental period. Rats, 150–200 g. body weight at the start of the experiment.)

Time after irradiation (days)	Bone marrow (mg. P/100 g.)			Spleen (mg. P/100 g.)			Testes (mg. P/100 g.)		
	DNA	RNA	DNA/RNA	DNA	RNA	DNA/RNA	DNA	RNA	DNA/RNA
Untreated*	157	81	1·94	100	66	1·51	23	41	0·56
4	15	23	0·65	37	42	0·88	20	35	0·57
14	58	56	1·04	98	83	1·18	28	45	0·62
21	79	71	1·12	106	87	1·22	18	33	0·54
48	169	91	1·86	99	60	1·65	20	26	0·77
Untreated†	165	79	2·07	94	54	1·74	27	43	0·63

* At beginning of experiment. † At end of experiment.

Table 11. *Late effects of body exposure to X-rays* (500 *r.*) *on the nucleic acid phosphorus content of rat bone marrow*

(Rats weighing 170–190 g. at start of experiment.)

Time after irradiation (weeks)	General condition	Total nucleic acid (mg. P/100 g.)	Total nucleic acid (% loss)	DNA (mg. P/100 g.)	DNA (% loss)	RNA (mg. P/100 g.)	RNA (% loss)	DNA/RNA
Untreated		267	—	179	—	88	—	2·0
1	Moribund; widespread haemorrhages	82	69	25	86	57	35	0·43
	Fair; slow gain of weight	215	19	130	27	85	4	1·53
2	Poor; loss of weight, offensive diarrhoea	128	52	64	64	64	27	1·00
	Poor; baldness, loss of weight	203	24	127	29	76	14	1·67
5	Fair; greyness, baldness, steady gain in weight	226	15	150	16	76	14	1·97
	Fair; greyness, sperms immotile	249	7	160	11	89	0	1·79
10	Fair; sperms immotile, growth of pigmented hair in bald areas	237	11	153	14	84	5	1·82

In several of these rats the liver content of glutathione and ascorbic acid as well as of glycogen was estimated during the early post-irradiation period, but no clear-cut results have so far been obtained. Livers of animals which succumbed to X-ray action with haemorrhagic manifestations contained no glycogen at all, but this may well have been due to poor food consumption.

Although rabbits were on the whole rather unsatisfactory experimental animals owing to the variability of their bone marrow P content, several experiments were carried out with litter mate rabbits belonging to the same breed. In Table 12 findings

DISCUSSION

Few data are available concerning the phosphorus content of bone marrow. The extensive and careful investigations of Davidson *et al.* (1947, 1948, 1950) deal with the content of nucleic acids in human bone marrow, obtained from normal individuals as well as from persons suffering from diverse forms of blood dyscrasias. It is not easy to compare results concerning bone marrow from the long bones of animals such as rats and rabbits with analyses based on human material aspirated from the sternum. Samples withdrawn by sternal puncture are likely to

Table 12. *Effect of body exposure to X-rays on the content of ATP and nucleic acid phosphorus in rabbit bone marrow*

(Two rabbit litters, *A* and *B*, of the same breed; irradiated with 1000 r.)

Litter	Time after irradiation (days)	Gross and microscopic appearance of bone marrow	ATP (mg. NH_2-N/ 100 g.)	Nucleic acid (mg. P/ 100 g.)
A	Untreated	Normal, very cellular	1·73	89
	2	Slight, admixture of blood; mitotic abnormalities	1·12	51
	4	Gelatinous and oedematous; severe cell damage	0·52	12
	10	Oedematous and pale; signs of cellular recovery	0·82	23
B	12	Pale and 'fatty'; signs of cellular recovery	0·68	42
	16		0·70	54

Table 13. *Distribution of phosphorus compounds in rabbit bone marrow 3 hr. after total body exposure to X-rays (1500 r.)*

Litter mates	ATP amino N (mg./100 g.)	Acid-soluble P (mg./100 g.)		Nucleic acid P (mg./100 g.)	
		$(P_{10} - P_0)$	$P_{tot.}$	DNA	RNA
Irradiated	0·18	3·0	28·6	27	15
Untreated	0·66	4·4	29·6	48	18

are recorded dealing with the behaviour of ATP and nucleic acid P after total body exposure to 1000 r. The animals showed few signs of distress in response to this dose, but there was a distinct decrease in the content of both these phosphorus compounds in the bone marrow, especially between the 2nd and 4th day; in this particular experiment even at 10 days values continued low as compared with those of the untreated litter mate, but in several other instances a nearly normal content of phosphorus was found at that stage. So far, experiments with rabbits have not been extended beyond 20 days; at that time there was a clear indication in some animals of complete, in others of partial, recovery of the bone marrow, both from the chemical and cytological point of view. A few experiments were carried out in which rabbits received larger doses of radiation (1500 r.) and the bone marrow was examined a few hours later, when the animals showed general symptoms of distress, some having intestinal disturbances; one such experiment is recorded in Table 13; ATP and nucleic acid P were already affected 3 hr. after irradiation, whereas total acid-soluble P remained unchanged.

contain a certain unavoidable and variable proportion of blood, whereas material from well bled animals usually has a negligible admixture of blood. Apart from technical details, anatomically distinct bones are known to contain marrow of different cellular compositions. But the greatest obstacle to comparison arises from the species differences in the make-up of the blood-forming tissue, such as were brought out by this investigation. The total nucleic acid P content of normal human marrow is given as 20·7 mg./100 g.; this is several times less than in rabbit, and about ten times less than in rat bone marrow. The DNA/RNA ratio in the bone marrow of normal rats varied within relatively narrow limits from 1·6 to 2·0; human material appears to show a wider range, 0·5–2·3, the average (1·3) being below that found in the animal species so far investigated.

Some determinations of the phosphorus content of rabbit bone marrow have also been described by Dietz (1949). However, his technique of phosphorus estimation was such as to render impossible interpretation in terms of the phosphorus fractions accepted for the experiments presented here. In the paper referred to the phosphate was determined as

follows: the bone marrow sample was first ground for an unspecified length of time in a mortar, then mixed with 5 vol. of water, and allowed to stand at room temperature for 1 hr.; after this trichloroacetic acid was added and in the protein-free filtrate 'phosphate' was precipitated as strychnine phosphomolybdate and determined by means of the colorimetric method used by Tisdall (1922) for phosphate estimations in serum. However, in serum total acid-soluble and directly determinable P are nearly identical, whereas in the bone marrow, in addition to small amounts of inorganic phosphate, there are several organic acid-soluble phosphorus derivatives, the content of which can only be assessed after incineration. Quite apart from that, 1 hr. incubation period between the collection of the tissue and the phosphate assay leads to changes in the pattern of phosphorus distribution owing to the presence in bone marrow of enzymes which act upon various phosphorus compounds of the tissue. There is, therefore, no common ground for the comparison of phosphorus in the protein-free filtrate from rabbit bone marrow. However, Dietz's values for 'total P' (obtained after incineration of whole tissue) do in some instances agree with those reported in this study. It would seem, however, that on the whole the assay of a more strictly defined phosphorus fraction, such as for instance nucleic acid P, is preferable as a basis for comparative studies in large numbers of animals to determinations of the 'total P', which, being made up of acid-soluble phospholipin, phosphoprotein and nucleic acid P, can be expected to vary widely.

The usefulness of the nucleic acid P value as an indicator of the metabolic potential of bone marrow was illustrated in experiments with myelotoxic agents such as mustard gas and X-rays, as well as in those which showed the influence upon the bone marrow of qualitative and quantitative changes in the food composition. The severe initial clinical symptoms caused by the toxic agents were accompanied by a decrease in the content of nucleic acid P in the bone marrow; as reported earlier (Lutwak-Mann & Gunz, 1949), this coincided with markedly lowered respiration and glycolysis of the bone marrow; in the surviving animals, parallel with improvement in the general condition, a gradual return to near normal values was observed in nucleic acid P; manometric measurements also indicated recovery in the respiratory and glycolytic functions of the bone marrow. Separation of nucleic acid into DNA and RNA made it possible to assess the relative sensitivity of these two fractions towards the damaging factors; judging from the DNA/RNA ratio it would seem that as a result of whole body exposure to X-rays, or in systemic mustard gas poisoning, DNA suffered more than RNA; the ratio fell sharply in the early experimental stage, but gradually returned to nearly normal values in animals which withstood the damage. It is, however, conceivable that other experimental conditions, for example, multiple small doses of radiation, or radiation applied more specifically to bone marrow-carrying areas and not to the body as a whole, would provoke in the blood-forming tissue a reaction which would result in a different pattern of distribution of these phosphorus compounds. The possibility must also be envisaged of pathological changes in the bone marrow which would find their expression, e.g. in reduced ability to form pigment without, perhaps, much change in cellularity and nucleic acid concentration.

Although nutritional deficiency considerably lowered the level of total nucleic acid P in the bone marrow, it has not yet been possible to establish any accompanying characteristic change in the DNA/RNA ratio. It is highly probable that deficiencies other than lack of vitamins of the B group would also lead to changes in the bone marrow; wider studies will perhaps bring out more clearly which of the two nucleic acids is more affected by dietary insufficiency.

During autolysis of bone marrow *in vitro*, RNA appeared to be more susceptible than DNA to enzymic attack, with the result that the DNA/RNA ratio became nearly double in the course of a few hours' incubation at 38°. The source of the ammonia which appeared at the same time has not yet been established; while it is certain that only a small proportion of this ammonia could have been derived from preformed ATP, it is not unlikely that some at any rate was connected with the breakdown of nucleic acids, more particularly of RNA. The processes observed during *in vitro* incubation of bone marrow were not limited to this tissue, as they were found to occur in spleen, though to a lesser extent.

The pharmacodynamically interesting experiments showing the potentiation of the effect of small, *per se* ineffectual doses of *HO* by DMT and DET, thiourea and urethane, lack for the time being adequate explanation of the mechanism involved, though the formation *in vivo* of *H* or a substance with *H*-like properties is very probable. In an attempt to approach this problem, several (unpublished) *in vitro* experiments were set up to see whether mixtures of *HO* with the thiocarbamates would inhibit bone marrow glycolysis in a manner similar to *H*; also mixtures of varying amounts of *HO* and DMT were first incubated with liver and kidney slices, and then bone marrow glycolysis was measured in these incubation fluids; however, it was not possible to reproduce *in vitro* conditions resembling those in the whole animal. Moreover, as reported above, the breakdown of bone marrow nucleic acids *in vitro* was not influenced by *H*, *HO*, or *HO*+DMT. In this connexion it is of interest to recall the work of

Peters & Wakelin (1947), who found that the *in vitro* inhibitory effect of mustard gas, and other vesicants with active chlorine atoms, towards the pyruvic oxidase system could be substantially increased by DET. This was found to be due to the formation of 2-chloroethyl-2-(*N*-diethyldithiocarbamato)ethyl sulphide, which was isolated in crystalline form, but which, however, had no toxicity in rats or guinea pigs.

From the practical point of view it is worthy of note that urethane (to which rats have been found to be remarkably resistant even on prolonged administration) caused a decline in nucleic acid P in the bone marrow, if combined with small, non-toxic amounts of *HO*; to establish whether the application of urethane 'fortified' with *HO* is suitable for clinical purposes, e.g. in the treatment of leukaemia, would necessitate extensive further laboratory as well as clinical trials.

SUMMARY

1. A study has been made of the phosphorus content in the bone marrow of rabbits and rats, with special reference to acid-soluble, adenosinetriphosphate, and nucleic acid phosphorus. The effect of certain experimental conditions upon the distribution of the various phosphorus fractions has been investigated, both in the whole animal and *in vitro*.

2. During *in vitro* incubation, enzymes present in the bone marrow caused several changes in the distribution of acid-soluble and nucleic acid phosphorus. There was a decrease in difficulty hydrolysable phosphorus esters accompanied by an accumulation of inorganic phosphorus; an increase in total acid-soluble phosphorus, particularly marked in rat bone marrow, and a fall in nucleic acid phosphorus, chiefly at the expense of ribonucleic acid; adenosinetriphosphate was relatively stable under aerobic conditions, but broke down more rapidly in absence of oxygen. Considerable amounts of ammonia, which exceeded many times the content of preformed adenosinetriphosphate amino nitrogen, were formed, especially in rat bone marrow.

3. Alterations in food composition leading to undernourishment or vitamin deficiency in rats markedly lowered the content of nucleic acid phosphorus in the bone marrow.

4. The behaviour of phosphorus compounds in the bone marrow was investigated at various stages of systemic poisoning with mustard gas and mustard sulphoxide, and also in rats and rabbits which received single doses of X-rays to the whole body. As a result of exposure of animals to these agents, there was a pronounced fall in nucleic acid phosphorus in the bone marrow; changes in the deoxyribonucleic/ribonucleic acid ratio suggested that deoxyribonucleic acid was more strongly affected than ribonucleic acid; a lowered content of adenosinetriphosphate and total acid-soluble phosphorus was also observed. In surviving animals, parallel with general recovery, there was a gradual return to normal in these phosphorus fractions of the bone marrow.

5. The significance of nucleic acid phosphorus assay as an indicator of the metabolic potential of the bone marrow is discussed.

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Phosphorus Compounds in the Cell

1. PROTEIN-BOUND PHOSPHORUS FRACTIONS STUDIED WITH THE AID OF RADIOACTIVE PHOSPHORUS

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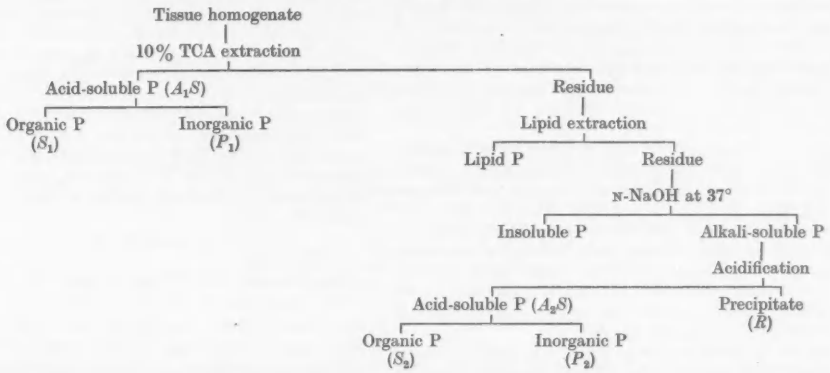
It has previously been shown (Brues, Tracy & Cohn, 1944; Hammarsten & Hevesy, 1946; Davidson, 1947) that when nucleic acids are isolated from the tissues of animals to which radioactive phosphorus (^{32}P) has been administered, the specific activity of the ribonucleic acid phosphorus (RNAP) is much greater than that of the deoxyribonucleic acid phosphorus (DNAP). These measurements have been carried out on ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) which have been isolated in a purified state from animal tissues (mainly the liver), but such a procedure requires a larger amount of tissue than is commonly available from small laboratory animals. It was therefore thought desirable to follow the distribution of radioactive phosphorus in the fractions separated by some such scheme as that of Schmidt & Thannhauser (1945), to determine whether isolation of nucleic acids in a pure state was in fact necessary, and to investigate phosphorus compounds other than the nucleic acids. Preliminary accounts of this work have already appeared (Davidson, Gardner, Hutchison, McIndoe, Raymond & Shaw, 1949; Davidson, Gardner, Hutchison, McIndoe & Shaw, 1949).

METHODS

Each animal (rabbit or rat) received radioactive P (^{32}P as inorganic phosphate; 100 μc./kg. body weight) by subcutaneous injection between the shoulder blades. After an interval of usually 2 hr. the animals were killed either by cervical dislocation or by exsanguination under ether anaesthesia. The abdomen was quickly opened and the liver perfused with warm 0·9 % NaCl solution through the portal vein. The organs and tissues required were rapidly removed, weighed, and chilled in ice. They were then homogenized in a Waring blender with an ice jacket, or in a Nelco blender, in 4 (or 9) vol. of ice-cold distilled water or 0·9 % NaCl solution. For small organs, such as the spleen, the entire homogenate was taken for analysis. With larger organs, e.g. the liver, an appropriate portion such as 20 ml. corresponding to 4 (or 2) g. tissue was employed. The homogenate was then submitted to the slight modification of the Schmidt-Thannhauser (1945) method of fractionation shown in Table 1. One half vol. of 30 % (w/v) A.R. trichloroacetic acid (TCA) was added to the homogenate which was then allowed to stand for at least 30 min. in ice. In some of the later experiments ^{32}P in 30 % TCA was added to homogenate of tissues from animals which had received ^{32}P 2 hr. previously or from control animals which had received no ^{32}P. The precipitated material was then centrifuged down and washed with ice-cold 10 % (w/v) TCA. In the initial experiments two washings with TCA were

Table 1. *Scheme of separation of phosphorus compounds in tissues*

(Slightly modified from Schmidt & Thannhauser, 1945.)



employed; subsequently as many as seventy washings were employed using a refrigerated centrifuge. The supernatant fluid and washings were set aside on ice for further fractionation as the acid-soluble P fraction (A_1S). The precipitate was extracted successively with acetone, ethanol, $CHCl_3$, ethanol-ether (3:1) (twice), and ether, and the extracts combined. These extracts were then taken to dryness on the water bath and the dry residue extracted with $CHCl_3$ to provide the lipid fraction (lipid P). A small amount of material in the lipid extract remained insoluble in $CHCl_3$, but contained a small amount of P.

The dry residue which remained was treated with a volume of N-NaOH equal to that of the original homogenate and was then incubated overnight at 37°. It was invariably found, contrary to the experience of Schmidt & Thannhauser (1945), but in agreement with observations of Mann & Gruschow (1949), who ignored the phenomenon, that the alkaline digest was very slightly turbid owing to the presence of a minute amount of insoluble material. This was usually filtered off through a hardened paper (Whatman no. 50), the process of filtration which took up to 24 hr. being accelerated by warming to 37°. The small precipitate was washed with distilled water and set aside for analysis (insoluble P). A 30 ml. portion of the alkaline digest plus the washings (alkali-soluble P) (total vol. 40 ml.) was then treated with 1·5 ml. of 10 N-H_2SO_4 and 20 ml. ice-cold 30 % (w/v) TCA. The precipitate containing proteins and DNA was centrifuged down and washed twice with small quantities of 10 % TCA. The final residue was dissolved in N-NaOH to yield fraction R. The supernatant fluid and washings, containing, among other things, the ribonucleotides formed by the breakdown of RNA, were combined and set aside as the second acid-soluble P fraction (A_2S).

Both acid-soluble fractions A_1S and A_2S contained inorganic phosphate, derived in the former from the inorganic phosphate of the tissue, and in the latter presumably from phosphoprotein (Plimmer & Bayliss, 1906). A portion of each extract was therefore taken for precipitation of inorganic phosphate as $Mg(NH_4)PO_4$. To such portions Mathison's (1909) reagent was added (1 ml. to 10 ml. extract) and the mixture made alkaline with NH_4OH. After standing overnight in the refrigerator the precipitate (which was minute and almost invisible in the case of the material from A_2S) was filtered off through a Whatman no. 42 paper, washed with dilute NH_4OH and dissolved in N-HCl. This provided the inorganic phosphate fractions P_1 and P_2 respectively. The mother liquors were taken as the organic phosphate fractions S_1 and S_2 respectively. An outline of the scheme of separation is shown in Table 1. Suitable portions of all fractions (in some cases the entire fractions) were taken for the determination of P by the method of Allen (1940) suitably modified for small quantities where necessary.

In some instances the separation procedure of Schneider (1945) was also used. The homogenate (20 ml.), corresponding to 4 g. of tissue, was submitted to extraction with TCA and lipid solvents as already described. The dry and finely powdered residue was then extracted for 15 min. with 5 % TCA at 90° to remove nucleic acids, leaving a P-containing protein residue. The precipitate was washed twice with 5 % TCA, supernatant fluid and washings being combined as the 'Schneider extract' and the final residue being dissolved in N-NaOH as the 'Schneider residue'.

For the determination of radioactivity, portions of each solution were pipetted into a liquid counter (Type M6 manufactured by 20th Century Electronics) attached to a conventional probe unit and scaling unit (Type 200; manufactured by Dynatron Radio Ltd.). All specific activities were calculated as counts/min./100 μg. P.

Isolation of nucleic acids

Since RNA is mainly cytoplasmic in origin while DNA is wholly nuclear, the isolation of each nucleic acid free from contamination by the other is clearly facilitated by a preliminary separation of cytoplasmic from nuclear material. Accordingly, a portion of liver homogenate in 0·9 % NaCl was centrifuged at low speed for 5 min. The turbid supernatant fluid was treated with 2 vol. of acetone and the precipitate centrifuged down, washed with ethanol and ether and dried. It was used for preparation of RNA.

The precipitate of nuclei and cell debris was washed twice with ice-cold 0·9 % NaCl and was then treated with acetone, ethanol and ether and dried. It was used for isolation of DNA.

RNA. The dried cytoplasmic material was extracted three times for 1 hr. each with 40 ml. of 10 % (w/v) NaCl solution at 100°. The combined extracts were treated with 2 vol. of ethanol and the precipitate of sodium nucleate was centrifuged down and washed with ethanol and ether. It was then dissolved in 10 ml. of water and the small insoluble residue was discarded. The supernatant fluid was treated with 2 vol. of glacial acetic acid and the precipitate so obtained was collected, washed with ethanol and ether and dried. The dry powder was dissolved in a small volume of ice-cold water with the cautious addition of 0·1 N-NaOH in the cold. The solution was made just alkaline to phenol red; a small insoluble residue was centrifuged down and discarded. Traces of protein were removed from the supernatant fluid by shaking with a small amount of $CHCl_3$-octanol mixture (4:1) and centrifuging. The aqueous supernatant layer was poured into 10 vol. of glacial acetic acid, the precipitated nucleic acid being centrifuged down, washed with ethanol and ether and dried.

DNA. The dried nuclear material was extracted for three successive 1 hr. periods in the boiling-water bath with 10 ml. portions of 10 % NaCl. The combined extracts were treated with 2 vol. of ethanol and the precipitate so obtained was centrifuged down, washed with ethanol and ether and dissolved in 10 ml. water. A small insoluble residue was centrifuged down and discarded. The supernatant fluid was then treated with 2 vol. of ethanol and the precipitate collected and dissolved in 2 ml. of N-NaOH. The solution was incubated overnight at 37° to hydrolyse any RNA present and was then treated with 2 ml. of 2 N-HCl and 2 vol. of ethanol. The precipitate was collected, washed with ethanol and ether, dried and dissolved in about 5 ml. of water with the cautious addition of 0·1 N-NaOH. $CHCl_3$-octanol (4:1) (0·5 ml.) was added and the mixture shaken and centrifuged. The supernatant fluid was poured from the protein gel into 5 vol. of a mixture of 2 ml. of conc. HCl and 10 ml. of ethanol, and the precipitate so obtained was washed with ethanol and ether and dried.

RESULTS

A large number of different organs and tissues from different species has been examined, and the results have been found to follow the same general pattern. The results of typical experiments on rabbit liver and spleen are shown in Tables 2 and 3 respectively.

Table 2. *Distribution of phosphorus and radioactivity in rabbit liver 2 hr. after injection of* ^{32}P

(Fractionation by modified Schmidt-Thannhauser procedure. Rabbit wt., 2200 g.; liver wt., 59 g.; dose of ^{32}P, 220 μc. by subcutaneous injection.)

Fraction	Phosphorus content (mg./100 g. fresh tissue)	Specific activity (counts/min./100 μg. P)
A_1S	142·7	2470
S_1	109·4	1572
P_1	32·0	4933
Lipid P	116·1	127
Alkali-soluble P	88·2	214
Insoluble P	2·5	331
A_2S	66·4	252
S_2	65·7	225
P_2	0·89	818
R	20·4	24

Table 3. *Distribution of phosphorus and radioactivity in rabbit spleen 2 hr. after injection of* ^{32}P

(Fractionation by modified Schmidt-Thannhauser procedure. Rabbit wt., 2480 g.; spleen wt., 1·6 g.; ^{32}P dose, 250 μc. by subcutaneous injection.)

Fraction	Phosphorus content (mg./100 g. fresh tissue)	Specific activity (counts/min./100 μg. P)
A_1S	76·9	2539
S_1	52·5	1564
P_1	22·3	3754
Lipid P	33·1	44
Alkali-soluble P	105·0	284
Insoluble P	2·7	321
A_2S	52·5	456
S_2	49·0	457
P_2	1·04	1101
R	51·2	53

It is seen that after 2 hr. the inorganic phosphate P_1 shows the highest activity of all, and contributes to the high activity of the A_1S fraction, although the organic phosphorus (S_1), which makes up the rest of this fraction and consists chiefly of phosphate esters, is also highly active. Of the remaining fractions the P_2 fraction (the 'phosphoprotein phosphorus'), although present in amounts of the order of only 1 mg./100 g. of liver, has an activity which is much higher than those of fractions S_2 and R in which the phosphorus is predominantly derived from the nucleic acids. The activity of the P_2 fraction is responsible for the high activity of fraction A_2S which is almost invariably higher than that of either of the nucleic acid fractions.

The insoluble P, i.e. the phosphorus contained in the insoluble material present in the alkaline digest, is also low in amount, but its activity is usually high, though not so high as that of the P_2 fraction.

Table 4 shows a comparison for rabbit liver of the activities of fractions S_2 and R obtained by the Schmidt-Thannhauser separation procedure with those of nucleic acids isolated from the same samples of tissue. The results show a wide scatter, but the activity of fraction S_2, which contains the ribonucleotides derived from RNA, is invariably higher than that of isolated RNA, while that of R is higher than that of isolated DNA. The discrepancy in the latter case is not so great as in the former, so that the activities ratio S_2/R is in most cases higher than the ratio RNAP/DNAP.

In some of our experiments an additional portion of the original tissue homogenate was subjected to the separation procedure of Schneider (1945). Table 5 shows a comparison of the results obtained for rabbit liver by this method with those obtained by the method of Schmidt & Thannhauser (1945). The specific activity of the phosphorus in the Schneider extract, which should contain both types of nucleic acid or their hydrolysis products, should give a value intermediate between those found for the separate S_2 and R fractions in the Schmidt-Thannhauser method. Though this is generally approximately true, the phosphorus content is always less than that of S_2 plus R. The Schneider residue, on the other hand, shows a much higher phosphorus content than would be expected on the basis of 'phosphoprotein' alone. Since the activity of this fraction, though high, is invariably lower than

Table 4. *Specific activities of phosphorus fractions obtained from rabbit-liver homogenates by the Schmidt-Thannhauser method compared with the activities of nucleic acids isolated from the same material*

(Dose of ^{32}P, 100 μc./kg. body wt., 2 hr. before killing. Activities are expressed as counts/min./100 μg. P.)

Rabbit no.	Activities of fractions		Ratio of activities	Activities of isolated nucleic acids		Ratio of activities RNAP
	S_2	R	S_2/R	RNAP	DNAP	DNAP
26	281	15	18·7	57	7	8·1
29	173	32	5·4	54	3	18·0
32	487	61	8·0	105	11	9·5
33*	311	16	19·4	45	5	9·0
46	118	30	3·9	52	15	3·5
47	322	21	15·3	59	13	4·5
49	237	16	14·8	91	12	7·6

* Pregnant.

Table 5. *Comparison of distribution of phosphorus and radioactivity in fractions isolated from rabbit-liver homogenates by the Schmidt-Thannhauser and by the Schneider procedures*

(Dose of ^{32}P, 100 μc./kg. body wt., 2 hr. before killing. *P*, amount of phosphorus in mg./100 g. fresh tissue; *C*, specific activity of phosphorus in counts/min./100 μg. P.)

Method ...	Schmidt-Thannhauser						Schneider			
Fraction ...	S_2		P_2		R		Extract		Residue	
Rabbit no.	*P*	*C*	*P*	*C*	*P*	*C*	*P*	*C*	*P*	*C*
26	44·1	281	1·0	428	13·9	15	52·3	82	22·0	133
29	77·7	173	1·4	1336	12·1	32	65·9	127	24·3	423
32	82·6	487	1·0	566	14·3	61	40·5	281	80·5	393
33	74·2	311	0·8	2585	17·9	16	60·0	203	38·5	525
46	75·8	118	0·2	2008	14·3	30	68·1	108	68·5	306
49	71·4	237	3·3	1208	22·4	16	70·9	131	23·2	137
50	65·7	225	0·9	818	20·4	24	60·6	153	24·0	416

that of P_2 it would appear that the phosphoprotein phosphorus in the Schneider residue is diluted by phosphorus of lower activity. The amount of phosphorus in the Schneider residue, which is often casually labelled 'phosphoprotein phosphorus', was so much greater than the amount of phosphorus in the P_2 fraction that it was thought desirable to carry out a Schmidt-Thannhauser separation on the Schneider residue. Table 6 shows the results of an experiment in which the Schneider residue was dissolved in N-sodium hydroxide and incubated in the usual Schmidt-Thannhauser manner, thereafter yielding on acidification an acid-soluble fraction A_2S and a residue R_3. Fraction A_2S was divided by precipitation with Mathison's reagent into an organic phosphorus fraction S_3 and an inorganic phosphorus fraction P_3. All these fractions had widely differing specific activities, but fraction P_3, the true inorganic phosphate, was present in the same order of quantity as P_2 and had a similar specific activity. It seems probable therefore that in the Schneider residue only fraction P_3 corresponds to fraction P_2 in the Schmidt-Thannhauser procedure, i.e. protein-bound phosphorus which is liberated in the inorganic state on incubation with alkali.

Table 6. *Distribution of phosphorus and radioactivity in fractions obtained from rabbit-liver homogenates by application of the Schmidt-Thannhauser separation alone and in combination with the Schneider procedure*

Fraction	Phosphorus content (mg./100 g. fresh tissue)	Specific activity (counts/min./100 μg. P)
Schmidt-Thannhauser method		
Alkali-soluble P	59·4	79
Insoluble P	0·3	105
A_2S	47·7	170
S_2	47·0	156
P_2	0·4	1641
R	11·7	19
Schneider method		
Schneider extract	44·5	52
Schneider residue dissolved in alkali and fractionated by the Schmidt-Thannhauser procedure, to yield:		
Alkali-soluble P	11·6	359
Insoluble P	0·1	15
Acid-soluble fraction (A_3S)	10·8	404
S_3	9·9	369
P_3	0·2	1850
R_3	1·5	235

In our usual separation procedure the amount of fraction P_2 actually handled is invariably very small (of the order of 10 or 20 μg.) and yields a precipitate of magnesium ammonium phosphate which is scarcely visible. Since the error in estimating such minute amounts of phosphorus tends to be high, we have carried out one or two experiments in which the amount of liver tissue extracted was of the order of 100 g. instead of the usual 4 g. In such experiments the amount of P_2 obtained was about 1 mg. and could easily be estimated with accuracy. Its specific activity was as high as in experiments conducted on a much smaller scale.

In one set of experiments an attempt was made to follow the variations in the activity of fraction P_2 with time. Female albino rats weighing about 150 g. each received a dose of 10 μc. of radioactive phosphorus per 100 g. body weight intramuscularly into the right thigh. Groups of six animals each were killed 2, 4, 24 and 48 hr. after injection and the livers were submitted to the fractionation scheme shown in Table 1. The results of such an experiment are summarized in Fig. 1 in which the mean specific activities of the fractions A_2S, S_2, P_2 and R relative to that of fraction P_1 as unity are plotted against time. There is a very considerable scatter in the results obtained from different animals, but it is clear from the graph that there is no direct correlation between the specific activity of fractions P_1 and P_2. The graph also shows that there is a steady rise in the specific activity of fractions A_2S and S_2 relative

to that of fraction P_1, and that in the later stages of this experiment these activities actually exceeded that of fraction P_2.

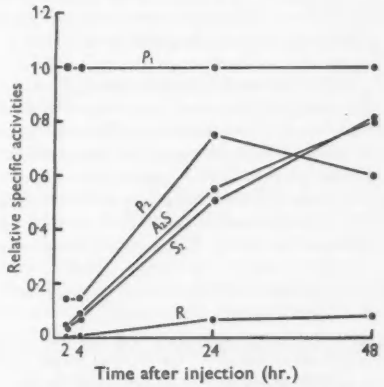


Fig. 1. Variations in the specific activities of phosphorus-containing fractions of rat-liver homogenates (relative to the activity of liver inorganic phosphate as unity) up to 48 hr. after intramuscular injection of ^{32}P (as inorganic phosphate). Schmidt-Thannhauser procedure. Each point represents mean value from six animals. (For the significance of P_1, P_2, A_2S, S_2 and R, see text, p. 312.)

It is generally considered that three or four extractions with TCA are sufficient to remove all acid-soluble material from a tissue homogenate, but we have thought it necessary to investigate this point in some detail. Table 7 shows the results of an experiment in which three equal portions of liver homogenate were extracted three, six and twenty times respectively with ice-cold 10% TCA. There is little difference between the results following three and six extractions, and in both cases the amounts and activities of fraction P_1 are of the same order. Twenty extractions, however, result in the removal of rather more phosphorus from the tissue homogenate, but from the eleventh to the twentieth extraction the specific activity of the phosphorus removed steadily falls to a value far below that of the main bulk of the acid-soluble fraction and indeed below that of most of the fractions containing phosphorus originally protein-bound. This suggests that the phosphorus removed in the later washings is not merely acid-soluble phosphorus which has failed to be extracted in the earlier washings, but that it is more probably derived from the breakdown of protein-bound phosphoric esters. It is important to note that fraction P_2 in all three cases is of the same order of amount and activity. Indeed the activity of the P_2 fraction from the tissue which has been extracted twenty times is not in any way diminished and is greater than that of the phosphorus extracted in the sixteenth to twentieth washings. These results suggest that fraction P_2 must contain inorganic phosphate other than that which has failed to be removed owing to inadequate extraction with TCA. Indeed, an experiment recorded in Table 10 shows that the amount of fraction P_2 is in no way reduced even after fifty-seven extractions with TCA.

While fraction P_2 appears to be bound to protein in such a way that it is released only on incubation with alkali, it is nevertheless possible that the specific activity of this fraction, and indeed of all the protein-bound fractions, may be unduly high owing to contamination with traces of inorganic phosphate derived from fraction P_1, and, as has already been mentioned, the results recorded in Table 4 strongly suggest this possibility. It should be emphasized that the specific activity of the inorganic phosphate

Table 7. *Comparison of the effects of extracting rat-liver tissue three, six and twenty times with* 10% *TCA*

(Dose of ^{32}P, 100 μc./kg., 2 hr. before killing. Schmidt-Thannhauser procedure. P, amount of phosphorus in mg./100 g. fresh tissue; C, specific activity in counts/min./100 μg. P.)

	Number of extractions with TCA					
	3		6		20	
Fraction	P	C	P	C	P	C
A_1S	107·5	5380	111·6	5120	111·4	5410
A_1S, 11th–15th extractions	—	—	—	—	2·9	1348
A_1S, 16th extraction	—	—	—	—	2·2	710
A_1S, 17th–19th extractions	—	—	—	—	5·3	481
A_1S, 20th extraction	—	—	—	—	1·0	189
S_1	66·8	3415	59·7	3490	64·9*	3910*
P_1	40·4	7450	41·3	7635	43·7*	7820*
Lipid P	108·1	871	106·0	891	117·0	874
Alkali-soluble P	125·5	372	129·7	348	128·2	334
Insoluble P	1·53	347	0·89	283	0·61	396
A_2S	97·7	444	100·1	404	97·5	399
S_2	93·3	470	97·5	403	95·4	383
P_2	0·12	1340	0·17	1195	0·20	1221
R	25·5	32	26·9	30	26·1	28

* Figures from 1st to 10th TCA extracts only.

fraction P_1 is so much greater than that of any of the protein-bound phosphorus fractions from the alkali-soluble P onwards, that contamination of any of these fractions with even a minute trace of fraction P_1 would result in a very considerable raising of their specific activities. In an attempt to achieve a more adequate removal of possible contaminating P_1 we have adopted a somewhat similar expedient to that suggested by Jeener (1949*b*) and by Friedkin & Lehninger (1949), of adding non-radioactive inorganic phosphate to minimize the activity of any inorganic phosphate still present after TCA extraction (Table 8). This procedure causes no appreciable alteration in the specific activities of the protein-bound phosphorus fractions.

In Table 8 also is shown the result of an experiment in which ^{32}P was added along with TCA to the homogenate of the liver tissue of animals which had received radioactive phosphorus by injection 2 hr. previously. Such artificially added inorganic radioactive phosphate might be expected to be completely removed by the extraction procedure with TCA so that the protein-bound phosphorus fractions should show the same radioactivity as is obtained from control tissues without such supplement. Table 8, however, reveals that this is by no means the case, and all the protein-bound phosphorus fractions, notably P_2 and R, show distinctly elevated specific activities. Similar results are shown in the experiment recorded in Table 9, in which even twenty

Table 8. *Effect of adding* $NaH_2{}^{31}PO_4$ *and* ^{32}P *as inorganic phosphate to homogenates of liver tissue from rats which had received* ^{32}P *2 hr. previously*

(*P*, amount of phosphorus in mg./100 g. fresh tissue; *C*, specific activity in counts/min./100 μg. P. Schmidt-Thannhauser procedure.)

	No addition		3 mg. ^{31}P added as NaH_2PO_4 to 4 g. tissue		0·5 μc. ^{32}P added to 4 g. tissue	
Fraction	*P*	*C*	*P*	*C*	*P*	*C*
A_1S	73·8	6725	145·4	3950	77·2	10420
S_1	28·9	5320	33·4	5110	31·5	5990
P_1	40·2	7860	117·1	4070	42·3	9690
Lipid P	76·8	743	75·1	928	84·8	782
Alkali-soluble P	102·6	452	111·5	433	108·7	486
Insoluble P	0·33	356	0·26	320	0·26	1915
A_2S	77·3	695	85·4	666	84·6	815
S_2	77·6	576	85·5	674	82·7	792
P_2	0·38	1465	0·54	1632	0·38	4410
R	24·3	41	24·3	45	24·1	215

Table 9. *Effect of adding* ^{32}P *as inorganic phosphate to homogenates of liver tissue from rabbits which had and which had not received* ^{32}P *by injection 2 hr. previously*

(*P*, amount of phosphorus in mg./100 g. fresh tissue; *C*, specific activity in counts/min./100 μg. P.)

Additions to homogenate	Injected animal* No addition				Injected animal* ^{32}P, 0·5 μc./4 g. tissue				Uninjected animal† ^{32}P, 0·5 μc./4 g. tissue			
Number of extractions with TCA	6		20		6		20		6		20	
Fraction	*P*	*C*	*P*	*C*	*P*	*C*	*P*	*C*	*P*	*C*	*P*	*C*
	Schmidt-Thannhauser procedure											
A_1S	145·2	4279	146·4	4625	142·7	6017	141·4	5942	128·3	2070	133·7	2048
S_1	107·3	3035	112·5	2962	107·3	3155	116·4	2926	102·2	120	109·2	142
P_1	25·4	8572	20·1	9190	26·8	17365	21·3	16600	21·1	9264	19·5	7525
Lipid P	61·0	165	76·9	161	66·3	194	80·6	238	50·1	45	47·8	35
Alkali-soluble P	73·6	407	72·1	521	78·4	1133	73·4	1238	45·8	1172	44·8	1113
Insoluble P	3·99	354	3·86	366	3·13	1553	2·65	1349	0·95	1103	1·94	1451
A_2S	57·2	522	57·9	482	63·9	1259	59·1	1295	39·2	1320	37·2	1286
S_2	56·5	462	56·2	404	58·4	945	56·2	1206	37·3	1238	35·5	1399
P_2	0·55	2191	0·57	1304	0·62	5069	0·48	2825	0·25	1458	0·25	856
R	12·0	139	12·8	122	13·0	667	13·2	649	6·1	768	6·1	592
	Schneider procedure											
Schneider extract	—	—	40·8	276	39·5	996	41·2	981	25·3	1030	24·8	1236
Schneider residue	—	—	30·7	589	37·5	1286	28·3	1580	16·1	1334	15·5	1587

* Female rabbit, wt. 2277 g., liver wt. 51·5 g.

† Male rabbit, wt. 2576 g., liver wt. 125·5 g. The exceptionally high weight of the liver of this animal may be related to the unusually low concentrations of P in all fractions from lipid P onwards.

extractions with TCA failed to remove inorganic radioactive phosphate added to a tissue homogenate along with the TCA. These results indicated such extreme difficulty in removing a serious contamination that we investigated the effect of adding ^{32}P as a minute amount of inorganic phosphate along with the TCA to the homogenate of liver tissue from an animal which had not received radioactive phosphorus. ^{32}P was mixed with TCA before addition to the homogenate in order to preclude the possibility of its enzymic incorporation into organic derivatives. The amount of ^{32}P added was such as was found to give a specific activity in fraction P_1 of the same order as that found 2 hr. after the injection of our customary dose of ^{32}P. It might reasonably be expected that if the extraction procedure were adequate, such contaminating inorganic radioactive phosphate would be completely removed during the extraction with TCA, but Table 9 shows that this is not so. As might be expected, the bulk of the radioactivity appears in fraction P_1 and only a little in fraction S_1 and in the lipid-P fraction. But whereas the protein-bound fractions from alkali-soluble phosphorus onwards should be free from radioactivity, most are in fact significantly radioactive. This contaminating radioactivity is not reduced when the number of extractions with TCA is increased from six to twenty (Table 9). It has been our experience that the activities of the protein-bound phosphorus fractions obtained in experiments of this type show much greater variation than those from animals which had received the ^{32}P by injection. When the Schneider procedure is adopted, similar contamination is also revealed; both the Schneider extract and the Schneider residue, which should not be radioactive, show considerable activity.

Table 10 shows the results of an experiment in which radioactive phosphorus was added along with TCA to a homogenate of liver tissue from an uninjected rabbit. The precipitate was subsequently extracted either with ice-cold 10% TCA or with 10% TCA containing 15% (w/v) of non-radioactive sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$). The extractions were carried out on a refrigerated centrifuge and were continued for several days in an attempt to obtain an extract with a count as low as that of the background. After forty-three extractions with TCA containing phosphate, fourteen further washings were carried out with 10% TCA to remove the added carrier phosphate. Even after this very exhaustive extraction the protein-bound phosphorus fractions still showed appreciable radioactivity, and it is clear from Table 10 that TCA containing phosphate is not much more effective in removing contaminating radioactive phosphorus than is TCA alone. Reprecipitation of the R fraction, however, has the effect of reducing both the amount of phosphorus, and the specific activity of that which remains after reprecipitation. From this it seems clear that the R fraction is not homogeneous as regards the phosphorus compounds present, even after more than fifty extractions of the protein material with 10% TCA.

We have also considered the possibility that addition of ^{32}P to the homogenate along with the first addition of TCA might produce an unusually persistent contamination of the protein precipitate, so giving rise to an exaggerated impression of the difficulty of freeing such precipitates from unwanted inorganic phosphate. In one group of experiments, therefore, we delayed the addition of ^{32}P until the first washing with TCA, i.e. until after the protein was fully precipitated. Table 10 indicates that while the resulting contamination of the protein fractions is slightly less than when the same amount of ^{32}P is added with the first quantity of TCA, it is, nevertheless, both extensive and persistent.

Table 10. *Effects of extraction with* 10% *TCA, and with* 10% *TCA containing* 15% $NaH_2PO_4.2H_2O$, *until extracts were free from radioactivity, on rabbit-liver tissue to which* ^{32}P *had been added along with TCA after homogenizing*

(P, amount of phosphorus in mg./100 g. fresh tissue; C, specific activity in counts/min./100 μg. P.)

	^{32}P added with 30% TCA				^{32}P added with first washing			
	57 washings with 10% TCA		43 washings with 10% TCA + 15% NaH_2PO_4, followed by 14 washings with 10% TCA		57 washings with 10% TCA		43 washings with 10% TCA + 15% NaH_2PO_4, followed by 14 washings with 10% TCA	
Fraction	P	C	P	C	P	C	P	C
Alkali-soluble P	57·0	57	59·6	51	57·2	44	56·8	39
A_2S	46·3	42	49·6	49	45·1	25	45·1	30
S_2	45·3	34	48·0	42	42·7	18	42·9	11
P_2	0·9	122	0·8	310	0·9	176	1·4	269
R	9·3	82	10·3	67	11·4	76	11·7	76
R^*	7·3	52	7·4	49	10·2	58	10·5	64

* Reprecipitated.

DISCUSSION

In the Schmidt-Thannhauser separation procedure the fraction which we have labelled A_2S contains acid-soluble ribonucleotides derived from the breakdown of RNA under the influence of warm alkali. Such compounds contain organically bound phosphorus, but the fraction also contains a small amount of inorganic phosphate. This is usually termed 'phosphoprotein phosphorus', since it is known from the work of Plimmer & Bayliss (1906) that the phosphorus of phosphoproteins is liberated in inorganic form by the action of alkali. 'Phosphoprotein phosphorus' is present in very minute amounts in most animal tissues, but usually tends to be completely ignored except in egg yolk and milk where it is abundant. Any objection that this fraction might consist merely of inorganic phosphate which has failed to be extracted during the preliminary washings with TCA can be dismissed in view of our observations that it persists in undiminished amount in tissues which have been extracted as many as fifty-seven times with 10% TCA.

In the Schneider (1945) procedure the tissue residue from which all acid-soluble phosphorus and lipid phosphorus have been removed is treated with hot 5% TCA to split off the nucleic acids as acid-soluble fragments, leaving a residue of protein-bound phosphorus which is usually assumed to be entirely derived from phosphoproteins. Schneider (1946) observed that this 'phosphoprotein phosphorus', as determined by his extraction procedure, was very much greater in amount than that which he found by the procedure of Schmidt & Thannhauser (1945), although he did not comment on the discrepancy. Our own observations amply confirm the fact that the Schneider 'phosphoprotein phosphorus' is many times greater than the Schmidt & Thannhauser 'phosphoprotein phosphorus'. That the Schneider residue contains other phosphorus compounds is shown by the fact that it has a very much lower specific activity than that of the 'phosphoprotein' fraction obtained by the Schmidt-Thannhauser method. This is supported by the results of our experiments in which the Schneider residue is dissolved in alkali and submitted to a Schmidt-Thannhauser separation. The inorganic phosphate fraction P_3 of Table 6 corresponds both in amount and in specific activity to the 'phosphoprotein' fraction P_2 found by the Schmidt-Thannhauser method. It is therefore clear that in any experiments on the protein-bound phosphorus fractions of tissues it cannot be assumed that the phosphorus of the Schneider residue represents only 'phosphoprotein', and it would appear that in the Schneider procedure the treatment with hot 5% TCA does not split off all the nucleic acid phosphorus.

In the Schmidt-Thannhauser separation, fraction A_2S contains ribonucleotides plus a small amount of inorganic phosphate from 'phosphoprotein'. Its pentose content, therefore, or better still the pentose content of fraction S_2 (on which pentose is more difficult to determine), should correspond in terms of phosphorus to that of a digest of RNA. This, however, is not the case. We have generally found that the pentose content of fraction A_2S corresponds to about 85–90% of the theoretical value for a digest of yeast RNA as calculated from phosphorus determinations. Similar figures have been found by Campbell & Kosterlitz (1949). It would appear probable, therefore, that fraction A_2S contains phosphorus compounds other than the acid-soluble ribonucleotides and the inorganic phosphate derived from 'phosphoprotein'.

It is clear from our results that protein-bound phosphorus fractions have a specific activity which is in general very much lower than that of acid-soluble phosphorus derivatives and is in consequence more liable to be greatly raised by traces of contaminating inorganic phosphate. Of the protein-bound phosphorus fractions, R has by far the lowest activity, but since even this low value is considerably higher than that of isolated DNA, contamination of the DNA in the R fraction with small amounts of phosphorus compounds of higher activity obviously occurs. As might be expected from results of early experiments on isolated nucleic acids, the specific activity of fraction S_2 is considerably higher than that of R. It is, however, clear that the S_2 fraction is also contaminated by phosphorus compounds of higher activity than that of isolated RNA.

The specific activity of fraction A_2S is in general slightly higher than that of fraction S_2 owing to the presence of the small amount of highly active phosphorus which later appears in the P_2 fraction. We have already commented on the particularly high activity of this fraction (Davidson *et al.* 1949) which has also been noted by Euler, Hevesy & Solodkowska (1948), by Friedkin & Lehninger (1949), and by Marshak & Calvet (1949). Juni, Kamen & Spiegelman (1948) have observed a high specific activity in the corresponding fraction from yeast, but in this case the fraction is very much more abundant than in animal tissues and consists essentially of metaphosphate. In confirmation of the work of Ebel (1949) we have failed to find any trace of protein-bound metaphosphate in animal tissues.

It might be considered that the so-called phosphoprotein fraction of the tissues, i.e. our fraction P_2, might arise not so much from true phosphoprotein, in which phosphate is esterified with hydroxy amino-acid residues, as from inorganic phosphate which had failed to be removed during the preliminary extraction with TCA. Such a possi-

bility has been kept in mind by Friedkin & Lehninger (1949), by Euler *et al.* (1948), and by Jeener (1949*b*), and in this connexion it is important to consider the number of extractions with TCA which is required to remove all traces of acid-soluble phosphorus. It appears to be the general practice to extract some three or four times with TCA and to assume that further washing will remove only negligible amounts of acid-soluble material. For example, McCarter & Steljes (1948) found that the last of four successive TCA extracts of 0·5 g. of rat-liver tissue contained only 0·006–0·008 mg. of phosphorus and concluded that further extraction was unnecessary. Our own extraction process has in many cases been much more exhaustive, and has revealed that even after fifty-seven extractions, using a refrigerated centrifuge, a small amount of phosphorus remains attached to protein and is released as inorganic phosphate only after incubation of the tissue residue with alkali. It appears probable, therefore, that our P_2 fraction does in fact arise mainly from true protein-bound phosphorus. Nevertheless, the possibility remains that the high specific activity of this fraction might be due to contamination with traces of inorganic phosphate which should have appeared in fraction P_1. This danger has been recognized by Friedkin & Lehninger (1949), by Euler *et al.* (1948) and by others. For example, McCarter & Steljes (1948) added radioactive phosphorus to a homogenate of rat-liver tissue and found that 96·5% of the added ^{32}P could be recovered in the acid-soluble extract. Since only minute amounts of the order of 0·005% were found in the nucleic acid fractions, they concluded that valid specific activity figures for these fractions were obtainable by the method employed (the Schmidt-Thannhauser procedure). This, however, is not necessarily the case since even minute contamination of the nucleic acid fractions with traces of inorganic radioactive phosphate might make very significant alterations to their normally low specific activities.

Dziewiatkowski & Bodian (1950), using mouse-brain tissue, found that four washings of their homogenate with 5% TCA were more than sufficient to remove all acid-soluble radioactive phosphorus, and that further extracts gave counts as low as that of the background. This has not been our experience with liver tissue, as can be seen in Table 7, from which it is evident that even the twentieth extraction with TCA is removing small amounts of phosphorus with appreciable radioactivity. It is evident from Table 7, however, that whether the number of extractions with TCA be three, six or twenty the overall picture of the specific activity of the protein-bound fractions is unchanged, and the activity of fraction P_2 is as high after twenty extractions with TCA as after 3. If the high activity of P_2 is in fact due to contaminating inorganic phosphate the contaminant is certainly not easily removed by further washing with TCA.

The extreme difficulty of removing the last traces of inorganic phosphate is abundantly clear from the results in Tables 9 and 10, where ^{32}P was added to the tissue homogenates along with the TCA. Not only does it appear that more than forty washings with TCA are required to obtain an extract with a count as low as that of the background, but even then the protein-bound fractions contain a significant amount of radioactive phosphorus which has failed to be removed by the previous exhaustive washing. Whether this phosphate exists in chemical combination with the proteins, or whether, as is more probable, it is physically adsorbed has not been determined, but it is clear from our results that the extent of the contamination is sufficiently serious to make it difficult to place any reliance on specific activity figures of fractions derived from protein-bound phosphorus.

It has been considered possible that the removal of contaminating inorganic radioactive phosphorus might be facilitated by repeated washing with non-radioactive phosphate. Friedkin & Lehninger (1949) employed three extractions with 10% TCA followed by three washings with 0·04 M-$NaH_2{}^{31}PO_4$ in TCA before applying the Schmidt-Thannhauser and Schneider procedures. Jeener (1949*a*, *b*), while investigating the fraction corresponding to our A_2S, recognized that the precipitation of the small amount of inorganic phosphate derived from 'phosphoprotein' might be incomplete, and could therefore give rise to unduly high specific activities in the S_2 fraction. He accordingly carried out repeated dilutions of the A_2S fraction with carrier inorganic phosphate and repeated precipitations with magnesia mixture. He found, however, that even the sixth washing with inorganic phosphate was still removing appreciable amounts of radioactive phosphorus.

Our own results shown in Table 10 reveal that repeated washing with inorganic carrier phosphate in TCA does not usefully aid in the removal of radioactive phosphorus.

In view of the difficulty of removing the last traces of inorganic phosphorus from precipitated tissue proteins it is not surprising to find (Table 4) that the specific activity of our fraction S_2 is invariably much greater than that of the phosphorus in isolated RNA and that the activity of our fraction R is greater than that of the phosphorus in isolated DNA.

There would therefore appear to be little justification for the assumption made by a number of authors, e.g. Campbell & Kosterlitz (1949), Holmes (1949*a*, *b*) and Hull & Kirk (1950*a*, *b*, *c*), that in order to obtain the specific activity of the nucleic acids in a sample of tissue from an animal to which ^{32}P has been given it is sufficient to carry out a Schmidt-

Thannhauser separation and to assume that the specific activity of the phosphorus in the A_2S fraction represents that of RNA while that of the phosphorus in the R fraction represents that of DNA. Not only does this assumption ignore the existence of the highly active P_2 fraction, which is of course included in A_2S, but it overlooks the complex nature of the fractions obtained in a Schmidt-Thannhauser separation which, in general, do not represent pure substances. As we have shown, the specific activities of the nucleic acids are invariably considerably lower than the specific activities of the fractions which contain them. The dangers of contamination have been recognized and stressed by Barnum, Nash, Jennings, Nygaard & Vermund (1950) and by Friedkin & Lehninger (1949) and especially by Jeener (1949*a*, *b*) and Jeener & Szafarz (1950*a*, *b*). The last named conclude that no reliance can be placed on the specific activities of nucleic acid fractions obtained by the Schmidt-Thannhauser method, and that the only way to determine the true specific activity is to isolate the nucleic acids in a rigorously purified form. Our own results lend ample support to this view, but we are by no means certain that any method of isolation so far described yields nucleic acids of the required standard of purity.

SUMMARY

1. The Schmidt-Thannhauser and the Schneider separation procedures have been applied to tissues, chiefly liver, from rats and rabbits injected with radioactive phosphorus (^{32}P), and the specific activities of the various phosphate fractions have been determined. The specific activities of the fractions containing the nucleic acids are invariably considerably higher than those of the nucleic acids isolated from the same tissues.

2. The 'phosphoprotein phosphorus', i.e. phosphorus set free from protein by incubation with alkali, invariably has a high specific activity. The 'phosphoprotein phosphorus' determined by the Schneider procedure is always much larger in amount but lower in activity than that obtained by the Schmidt-Thannhauser procedure owing to the presence of other phosphorus compounds in the Schneider residue.

3. Exhaustive extraction with ice-cold 10% TCA fails to remove 'phosphoprotein phosphorus' from liver tissue or to reduce its high specific activity. On the other hand, the high activity of this fraction may be due partly to persistent traces of acid-soluble phosphate, since ^{32}P added as inorganic phosphate to a tissue homogenate is not completely removed even by numerous washings with 10% TCA. Washing with TCA containing carrier phosphate is also inadequate.

4. It is concluded that it is unjustifiable to assume that the specific activities of the nucleic acid fractions obtained by the Schmidt-Thannhauser procedure represent the true activities of the nucleic acids. In all experiments on protein-bound phosphorus fractions involving the use of ^{32}P it is essential to eliminate the possibility of contaminating inorganic phosphate. To do this it is desirable to isolate the compounds under investigation in the pure state.

We wish to thank Mr J. E. McEwen, Mr J. Sommerville and Miss Joyce Leslie Smith for technical assistance in the course of these experiments, and we should also like to acknowledge with thanks an expenses grant to one of us (J. N. D.) from the Medical Research Council, a grant from the Rankine Fund of the University of Glasgow for a power unit and other equipment, and a grant from the Rockefeller Foundation of New York (to J. N. D.) for a refrigerated centrifuge.

Note added 2 *March* 1951. The experiments described in this paper were carried out with ^{32}P supplied by Atomic Energy Research Establishment, Harwell, which issued a statement in January 1951 that 'a number of solutions of phosphorus 32 dispatched from Harwell before the end of November 1950 have contained variable amounts of particulate matter.... In some solutions the activity is partially concentrated on these particles.' We have therefore repeated experiments of the general type shown in Tables 2–7 and 9 with two different batches of ^{32}P stated to be free from this defect and have found that the general pattern of activity in injected animals is as before. When ^{32}P was added with TCA to homogenates of liver tissue as in Table 9 we again found persistent radioactive contamination of the protein-bound phosphorus fractions. The conclusions arrived at do not therefore appear to be affected.

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The Hexokinase Activity of Animal Tumours

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Slices of normal and tumour tissue which convert glucose to lactic acid rapidly (Warburg, 1930) have but little action on hexosediphosphate (Boyland & Boyland, 1938). This difference is probably due to the fact that hexosediphosphate cannot diffuse into the tissue cells, but it was interpreted as an indication of non-phosphorylating glycolysis in tissues (Needham & Lehmann, 1937). The demonstration of glycolysis of glucose, fructose and hexosediphosphate by fortified tumour extracts at the same rate as glycolysis in tumour slices (Boyland, Boyland & Greville, 1937) indicated that glycolysis in tumours involved phosphorylation. No positive evidence of a glycolytic system in tumour tissue differing from that of other tissues has been put forward and enzymes of the normal glycolytic cycle have been found in tumour tissue. Meyerhof & Wilson (1949) showed that glycolysis in tissue extracts is dependent on the ratio of phosphorylating and dephosphorylating enzymes present, and that tumour extracts contain excessive amounts of adenosinetriphosphatase (ATP-ase) relative to the hexokinase content.

The facts presented in this paper show that the hexokinase activity of certain rat tumours is sufficiently great to allow all the glucose used in anaerobic glycolysis of the tumour slices to pass through the stage of the hexokinase reaction. Some properties of the hexokinase of various rat and mouse tumours have also been investigated.

METHODS

Preparation of homogenates. The animals were killed by a blow on the head, the tumour removed and necrotic material dissected away as quickly as possible. The tissue was then allowed to cool on ice for 10 min. A portion (2–3 g.) was chopped with scissors, weighed and immediately added to an all glass homogenizer (Potter & Elvehjem, 1936) and homogenized with 7 vol. of ice-cold 0·002 % glucose. The resulting homogenate was strained through a wisp of glass-wool to remove fibrous strands.

Preparation of acetone powders. The tumour was removed immediately after the animal was killed, dissected free of necrotic material and allowed to cool on ice for 10 min. The tissue was then homogenized in a Waring blender with 5 vol. cold acetone (− 5°) for 2–3 min. at 2°. The solid material was filtered off on a Büchner funnel and washed at least five times with cold acetone without allowing the filter cake to crack. It was then dried in a vacuum desiccator over $CaCl_2$. The dry powder, if kept *in vacuo* at 2°, retained its hexokinase activity for at least 1 month. Aqueous extracts of the acetone powders were prepared by triturating with 10 vol. of ice-cold glass-distilled water and then centrifuging at approx. 2000 *g* for 15 min. at 2°. The resulting supernatant fluid, after being passed through a wisp of glass-wool, was used for determination of the hexokinase activity.

Estimation of hexokinase activity. (*a*) *In homogenates.* The reaction was carried out in open test tubes at 38°. To each tube was added 0·50 ml. buffer; 0·10 ml. 0·2 M-$MgCl_2$; 0·10 ml. 1·5 % glucose and 0·50 ml. water and/or additions. Freshly prepared homogenate (1·00 ml.) was then added followed by 0·30 ml. M-KF (the fluoride was added after the homogenate had been added to the Mg-containing buffer mixture in order to minimize precipitation of MgF_2). The tubes were then incubated at 38° for 5 min. and the reaction started by the addition of 0·50 ml. adenosinetriphosphate (ATP) solution (containing approx. 2 mg. acid-labile P). Duplicate tubes were removed immediately after the addition of the ATP in order to give values for the initial glucose concentration. The other tubes were incubated at 38°, while being shaken at the rate of 120 cyc./min., for 10–20 min. The reaction mixtures were deproteinized by the addition of successive equal volumes of 0·3 N-$Ba(OH)_2$ and 5 % $ZnSO_4$;

after mixing and filtration, glucose was estimated in the filtrates either by the method of Nelson (1944) or that of Somogyi (1939). The hexokinase activity was expressed in terms of μmol. glucose used/g. dry wt. of tissue/min. The dry weights of the homogenates were determined by heating to constant weight in an oven at 110°.

(*b*) *In acetone powder extracts.* The activity was determined by the manometric method of Colowick & Kalckar (1943), the reactions being carried out in conventional Warburg flasks. The transfer of 1 mol. of phosphate from ATP to glucose results in the liberation of 1 acid equiv. The main compartment of the vessels contained 1·00 ml. M-$NaHCO_3$; 0·02M-$MgCl_2$; 0·10 ml. sugar solution; 0·20 ml. M-KF and the volume made up to 2·20 ml. with enzyme, water and additions. The side arm contained 0·10 ml. $NaHCO_3$; 0·02 M-$MgCl_2$ and 0·20 ml. ATP (containing approx. 1 mg. acid-labile P in most experiments). The gas phase was 95% N_2–5% CO_2. The bicarbonate concentration varied in different experiments, and is recorded under the relevant protocols. After 10 min. equilibration at 38° the contents of the side arm were tipped into the main compartment and the gas evolution measured at 5 min. intervals for the next 30–60 min. Blanks containing all the reactants except the sugar were run in order to give values for the gas exchange which occurred on tipping the ATP solution into the main compartment and also for the small ATP-ase activity of some of the extracts. Unless otherwise stated, all figures recorded are corrected for ATP-ase activity (in cases where they are not, the CO_2 evolution for the first 10 min. after tipping was ignored).

Reagents. ATP, hexosediphosphate (HDP) and cozymase (Co I) were obtained from Schwarz Ltd. Methylbis(2-chloroethyl)amine was obtained from the Ministry of Supply. Samples of *NN*-di(2-chloroethyl)-β-naphthylamine; 2:4:6-tris(ethyleneimino)-*s*-triazine and butadiene diepoxide were kindly donated by Dr W. C. J. Ross. D-Fructose was obtained from Hofmann-La Roche Ltd. and recrystallized from ethanol. D-Galactose was obtained from Thomas Kerfoot Ltd., D-mannose and D-glucose from British Drug Houses Ltd.

The hexokinase activity of freshly prepared tumour homogenates

Hexokinase activity was demonstrated in the presence of the following buffers: 0·025M-sodium bicarbonate; 0·015M-potassium phosphate, pH 7·4; 0·08M-tris(hydroxymethyl)aminomethane, pH 7·6 and tris(hydroxymethyl)aminoethane, pH 7·6. In the experiments performed in the presence of sodium bicarbonate the gas phase was 95% N_2–5% CO_2, while with the other buffers used the gas phase was air. Experiments carried out in the presence of tris(hydroxymethyl)aminomethane and also phosphate buffers showed that no glucose was used over an initial 15 min. period when ATP was omitted from the reaction mixture.

With homogenates of Walker 256 carcinoma prepared from 8- to 10-day-old tumours the hexokinase activity in phosphate and tris(hydroxymethyl)-aminomethane buffers varied from 13 to 25 μmol./g./min. Homogenates of the necrotic cores of older tumours were devoid of hexokinase activity. With fresh tumour homogenates the reaction rate was proportional to the amount of tissue added over the range of approx. 5–25 mg. dry weight per reaction mixture. Maximal rates of reaction were observed with a final ATP concentration of 0·01M (if the ATP concentration was raised to 0·03M the rate was slightly diminished, possibly due to impurities in the ATP preparations used). The hexokinase activity with 0·01M-ATP and 1·5 mg. glucose per reaction mixture was linear with time for at least 15 min. The experiments summarized in Table 1 show that in the presence of bicarbonate buffer the addition of potassium ions had little influence on the hexokinase activity.

Table 1. *Influence of* KCl *on the hexokinase activity of Walker* 256 *carcinoma homogenate*

(0·01M-$MgCl_2$; 0·025M-$NaHCO_3$; 0·05M-NaF; 0·009M-ATP. Gas phase: 95% N_2–5% CO_2.)

Concentration of added KCl (M)	Hexokinase activity (μmol. glucose utilized/g./min.)
0·0013	7·1
0·0076	7·5
0·014	8·0
0·026	7·9
0·051	8·0
0·100	7·4

The influence of the *in vitro* addition of a number of substances on the hexokinase activity of freshly prepared homogenates of Walker 256 carcinoma is shown in Table 2. The degree of inhibition found with methylbis(2-chlorethyl)amine and protoanemonin is similar to that observed with yeast hexokinase by Dixon & Needham (1946). Ethyl carbamate, without influence on the tumour hexokinase when added *in vitro*, was also devoid of significant influence on the hexokinase activity of Walker 256 carcinoma when administered *in vivo*. Six rats were implanted with

Table 2. *Inhibition of the hexokinase activity of freshly prepared homogenates of Walker* 256 *carcinoma*

(Values shown are percentage inhibition.)

Inhibitor concentration (M)	0·0006	0·0012	0·0025	0·005	0·01
Choline chloride	—	—	0	0	0
Methyl-bis(2-chloroethyl)amine	—	42	58	75	—
Protoanemonin (vinylacrylolactone)	61	78	100	100	—
Ethyl carbamate	—	—	—	—	0
Amidone (6-dimethylamino-4:4′-diphenylheptan-3-one hydrochloride)	—	16	19	17	—

the tumour and pair-fed on a vitamin-supplemented diet containing 5% protein. On the fifth day after implantation three animals were injected with 500 mg. ethyl carbamate/kg. while the controls were injected with water. The same doses were given daily for the following 4 days. The tumours were then removed from the animals and the hexokinase activity determined in phosphate buffer. No significant difference between the activity of the ethyl carbamate-treated animals and that of the controls was observed, despite the fact that the tumours of the former group were much smaller.

Homogenates of Jensen rat sarcoma exhibited the same order of hexokinase activity as those of Walker 256 carcinoma. One sample of Rous tumour (removed from the bird 28 days after inoculation of the virus) had a hexokinase activity of 13·4 μmol. glucose utilized/g./min.

The hexokinase activity of aqueous extracts of rat and mouse tumour acetone powders

Rapid phosphorylation of D-glucose was observed in the presence of extracts prepared from Walker 256 carcinoma, Jensen rat sarcoma, S37 mouse sarcoma and Crocker 180 sarcoma. The ATP-ase activity of such preparations was usually very small, and was never greater than 15% of the hexokinase activity. In the presence of glucose without ATP, no gas output whatsoever was observed. Hexose-diphosphate (final concentration 0·3%) gave rise to a carbon dioxide output no greater than that observed with ATP alone, hence the results obtained appeared to be uncomplicated by gas-producing reactions occurring past the phosphohexokinase stage. The hexokinase activity was proportional to the amount of enzyme solution over a fairly wide range (0·20–0·70 ml. of a 1:20 extract/vessel). Most experiments were performed with 0·4 or 0·5 ml. of enzyme solution in each flask.

In one experiment it was observed that 0·5 ml. of a 1:30 aqueous extract of an acetone powder of Rous tumour exhibited a small but definite hexokinase activity. However, the high viscosity of such extracts make them unsuitable for hexokinase determination.

D-Fructose and D-mannose, but not D-galactose, were also phosphorylated by the enzyme. The relative rates of reaction in the presence of 2 and 50 μmol. of glucose and fructose (final concentrations of 0·008 and 0·02M, respectively) with extracts prepared from different tumours is shown in Table 3. Over an initial 10 min. period the rate with 2 μmol. glucose varied between 85 and 100% of that in the presence of 50 μmol. glucose. Fig. 1 shows that the rate of reaction with 2 μmol. glucose paralleled that with 50 μmol. glucose until most of

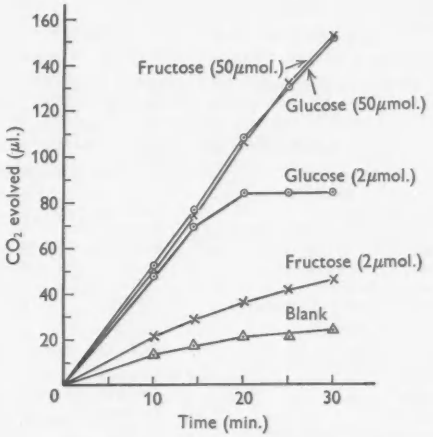

Fig. 1. Phosphorylation of glucose and fructose by Crocker 180 mouse tumour hexokinase.

the substrate had been exhausted. When 50 μmol. fructose were added per vessel, the hexokinase activity varied between 72 and 96% of that in the presence of 50 μmol. glucose, but with 2 μmol. fructose per vessel, the reaction rate varied between 3 and 21% of that in the presence of 50 μmol. fructose. The greater affinity of glucose as opposed to fructose for the enzyme is similar to that observed with tumour homogenates by Meyerhof & Wilson (1949) and with

Table 3. *Phosphorylation of glucose and fructose by hexokinases of various rat and mouse tumours*

(All experiments carried out with 0·40 ml. 1:20 aqueous extract of acetone powder. Total volume of reaction mixture = 2·50 ml.)

Tumour	Concn. of $NaHCO_3$ in medium (M)	pH*	Hexokinase† activity with 50 μmol. glucose/flask	Relative rates (50 μmol. glucose = 100) in presence of: 2 μmol. glucose	50 μmol. fructose	2 μmol. fructose
Jensen rat sarcoma	0·0265	7·65	39·0	85	72	3
Walker 256 carcinoma	0·042	7·83	15·0	100	—	10
S37 mouse carcinoma	0·042	7·83	33·5	91	90	21
Crocker 180 mouse carcinoma	0·042	7·83	38·5	87	96	17

* At 37°, calculated from $NaHCO_3$ concentration in equilibrium with 95% N_2–5% CO_2.
† CO_2 (μl.) evolved in 10 min. after tipping ATP, corrected for ATP-ase activity.

brain acetone powder extracts by Wiebelhaus & Lardy (1949). Extracts of Jensen rat sarcoma phosphorylated D-mannose at about 90% the rate of glucose (final concentration of both sugars 0·02M).

The hexokinase activity of Jensen rat sarcoma acetone powder extracts was not much increased by the addition of potassium ions. Thus, in a system containing no potassium salts, the addition of 0·08M-potassium fluoride in place of 0·08M-sodium fluoride enhanced the hexokinase activity some 20% (the ATP-ase activity was unaltered), but did not significantly affect the shape of the carbon dioxide output curve over the first 30 min.

The influence of the *in vitro* addition of a number of substances on the hexokinase activity of aqueous extracts of Jensen rat sarcoma acetone powder is shown in Table 4. Certain of the compounds used

Table 4. *Inhibition of tumour hexokinase*

Effector	Concentration (M)	Inhibition (%)
Ethyl carbamate	0·027	0
Ethyl *N*-phenylcarbamate*	0·0024	0
Methyl-bis(2-chloroethyl)amine†	0·004	62
NN-Bis(2-chloroethyl)-*β*-naphthylamine*	0·0002	0
2:4:6-Tris(ethyleneimino)-*s*-triazine	0·0003	0
Phlorrhizin	0·009	0
Butadiene diepoxide	0·01	15
$CuSO_4$	0·00005	66
$CoCl_2$	0·0001	12
Suramin‡	0·00008	43
Amidone (6-dimethylamino-4:4′-diphenylheptan-3-one hydrochloride)	0·0028	0

* Dissolved in ethylene glycol monoethyl ether.

† Corrected for hydrolysis; compound added to flasks immediately after solution.

‡ Symmetrical urea of the sodium salt of *m*-benzoyl-*m*-amino-*p*-methylbenzoyl-1-aminonaphthalene-4:6:8-trisulphonic acid.

Final concentration of bicarbonate buffer 0·0265M.

were but sparingly soluble in water, and were added to the flasks in 0·10 ml. ethylene glycol monoethyl ether (EGEE), the appropriate controls being run in each case. Other experiments demonstrated that this concentration of EGEE was devoid of significant influence on the hexokinase activity.

DISCUSSION

It is of interest to compare the observed rates of hexokinase activity in tumour homogenates with the known glycolytic rates of slices of such tumours. Since each molecule of glucose gives rise to 2 mol. of lactic acid, the hexokinase activities expressed in terms of μmol. glucose utilized/g. dry wt./min. must be multiplied by $2 \times 22{\cdot}4 \times 60/1000 = 26{\cdot}88$ to be converted into equivalent $Q^{N_2}_{CO_2}$ values in μl./mg. dry wt./hr. Thus the $Q^{N_2}_{CO_2}$ values equivalent to the hexokinase activity of Walker 256 carcinoma and Jensen rat sarcoma varied between 35 and 67. The maximal $Q^{N_2}_{CO_2}$ for slices of these tumours at 37° is approximately 35, so that the hexokinase activity appears to be at least sufficient to allow all the glucose utilized to pass through this reaction. Whether hexokinase is a possible rate limiting factor in tumour glycolysis, however, can only be decided after further experiments. In this study no measurements of the possible dephosphorylation of hexose phosphates under the conditions of the hexokinase assay were made, and it is possible that the hexokinase activity values observed are too low since they are computed from estimations of glucose utilization. Nevertheless, assuming that the hexokinase activities are not grossly in error, and that the enzyme is maximally active in slices, it would seem that the hexokinase content of tumours resembles that of aldolase (Warburg & Christian, 1943; Sibley & Lehninger, 1949) in being sufficient to allow a glycolytic rate not greater than 2–3 times that observed in tumour slices. Other glycolytic enzymes, e.g. triosephosphate isomerase (Oesper & Meyerhof, 1950) are known to be present in far greater excess in animal tumours.

The data given in Table 4 on the inhibition of tumour hexokinase by a number of mitotic poisons would suggest that, although nitrogen mustard is an inhibitor of hexokinase, this is not an essential character of mitotic poisons. The other radiomimetic mitotic poisons tested in this experiment (ethyl carbamate, *NN*-bis(chloroethyl)-*β*-naphthylamine, 2:4:6-tris(ethyleneimino)-*s*-triazine and butadiene diepoxide) are all substances which react relatively slowly (cf. Ross, 1950) so that chemical reaction between the inhibitor and enzyme would be negligible under the experimental conditions. Those experiments, therefore, give no evidence on the possible relation between mitotic inhibition and poisoning of hexokinase.

SUMMARY

1. The hexokinase activity of animal tumours is sufficient to account for all the glucose used in the anaerobic glycolysis of tumour slices passing through the hexokinase reaction.

2. The tumour enzyme phosphorylated glucose and mannose readily. For optimum phosphorylation of fructose a higher concentration was required. Galactose was not phosphorylated.

3. The enzyme was inhibited *in vitro* by methyl-bis(2-chloroethyl)amine but not by other radiomimetic mitotic poisons which react more slowly.

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The Utilization of Non-protein Nitrogen in the Bovine Rumen

7. A QUALITATIVE AND QUANTITATIVE STUDY OF THE BREAKDOWN OF CARBOHYDRATE WHICH ACCOMPANIES PROTEIN FORMATION IN BOVINE RUMEN CONTENTS DURING *IN VITRO* INCUBATION

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In an earlier paper of the present series Pearson & Smith (1943*a*) showed that when fresh rumen contents were incubated *in vitro* both synthesis and hydrolysis of protein could readily occur, but that to obtain the prolific bacterial growth which results in protein synthesis predominating over protein hydrolysis, an ample supply of readily available carbohydrate was essential. It would appear probable, therefore, that the non-protein nitrogenous constituents of feeding stuffs may be of value in the rumen only when there is also an abundance of simple sugar or starch in the diet, and since the products commonly fed to ruminants contain many different types of carbohydrates and related compounds, it seemed important to determine to what extent protein synthesis predominated in rumen contents when a variety of different substances were made available as possible sources of energy for the bacteria. It was hoped also that the work would give information regarding the types of compound on which rumen micro-organisms can act. Pearson & Smith (1943*a*) had already shown that when soluble starch, D-(+)-galactose, sucrose, maltose, lactose and D-(+)-glucose were added to rumen liquid during incubation *in vitro*, vigorous bacterial proliferation resulting in the predomination of protein synthesis occurred. The present communication describes the extension of that work to include other naturally occurring carbohydrates, such as arabinose and xylose, and also certain derivatives of carbohydrates. Several other substances including lower fatty acids, lactic acid and succinic acid have also been tested.

It is now well established that much of the carbohydrate in the diet of ruminating animals is fermented in the rumen to form various end products which include lower fatty acids, carbon dioxide and methane (Phillipson, 1947–8; Elsden & Phillipson, 1948), but since the rumen is part of an open system from which these products can pass further along the alimentary tract or can be absorbed into the blood or be excreted in the breath, it is not easy to estimate *in vivo* the proportions in which these various substances are produced. It seemed possible, however, that by adapting the *in vitro* incubation technique of Pearson & Smith (1943*a*) so that the gaseous products of fermentation could be collected and analysed, it would be possible to determine approximately, but with more accuracy than hitherto, the relative proportions in which the various end products were formed from different sugars, and how much of the utilized sugar could be accounted for by the end products. Since maltose and the pentoses arabinose and xylose are all known to occur as units of more complex carbohydrate molecules in vegetable products, these three carbohydrates were used in this quantitative section of the work.

The objects of the present investigation were first to find what types of compound could be utilized by the rumen bacteria to enable protein synthesis to predominate over protein hydrolysis, and secondly to determine the proportions in which the main end products of carbohydrate fermentation in rumen contents are formed, how much of the fermented carbohydrate can be accounted for by these pro-

ducts, and whether the end products with a disaccharide like maltose, which consists of hexose units, differ from those formed when the fermented sugar consists mainly of a pentose such as arabinose or xylose.

METHODS

The method of obtaining and incubating samples of fresh rumen contents from animals with a permanent rumen fistula has been described by Pearson & Smith (1943*a*). Since, however, Smith & Baker (1944) showed that the rumen protozoa appeared to play no part in the marked protein synthesis which they obtained in their brief *in vitro* incubations, the protozoa were removed from the rumen liquid by light centrifugation (5 min. at 2000 rev./min., approximately 800 *g*) before incubation began. Urea (0·05 %) was added to all samples of rumen liquid to ensure that lack of non-protein nitrogen (N.P.N.) did not in any way limit bacterial activity. Unless otherwise stated, carbohydrates and other test substances were added at a level of 1 %. The period of incubation in all experiments was 4 hr. For experiments in which CO_2 and CH_4 production were measured, the incubation flasks were fitted with air-tight bungs carrying inlet and outlet tubes, and a slow stream of CO_2-free air was drawn through the incubating material to remove the gaseous products to the aspirators in which they were collected. Further details are given later (p. 328).

The amount of protein synthesis occurring during incubation was taken as a measure of bacterial activity. Samples from the incubation flask were therefore preserved before and after incubation for chemical analyses.

Methods of analysis

Nitrogen. The N.P.N. and protein N of the rumen liquid were partitioned by the sodium tungstate procedure of Pearson & Smith (1943*c*).

Carbohydrates. The sample of rumen liquid was first separated into bacterial sediment and supernatant liquid by centrifugation as described by Smith & Baker (1944). The reducing power of these fractions before and after acid hydrolysis was then determined in order to obtain the amount of bacterial polysaccharide and soluble carbohydrate which each contained. For this purpose the gravimetric Cu_2O method of Munsen & Walker, as recommended by the Association of Official Agricultural Chemists (1945), was used. Where pentoses were being studied, the amount present in the bacterial sediment and supernatant liquid was measured by the furfural-phloroglucide procedure, as described by the Association of Official Agricultural Chemists (1945).

Total volatile fatty acids. These were estimated by exhaustive steam distillation after acidification with 10 N-H_2SO_4. In a typical experiment 50 ml. of rumen liquid were acidified with 10 ml. of 10 N-H_2SO_4 and about 1 l. of distillate collected. The distillate was titrated with 0·1 N-NaOH and the acidity expressed in terms of acetic acid.

Volatile fatty acid partition. The acids were first separated from the rumen liquid by the double distillation procedure of Friedemann (1938). The silica-gel partition chromatographic technique of Moyle, Baldwin & Scarisbrick (1948) was used to separate the acids into acetic, propionic and butyric acid fractions. The last fraction contained a very small amount of a higher acid which was not identified.

Lactic acid. The method of Barker & Summerson (1941) was found to be satisfactory and was adopted.

Gas analysis. When samples of gas were collected from the rumen incubations they were analysed for CO_2 and CH_4 in a Haldane apparatus.

RESULTS

The bacterial activity in rumen liquid in the presence of carbohydrates, derivatives of carbohydrates, lower fatty acids and other compounds

In this section of the work a sample of rumen liquid, prepared as already described, was divided into the required number of portions. To one portion, which served as a control, 1 % maltose was added; to the others 1 % of a test substance was added. In some experiments one portion of the rumen liquid was incubated without addition of carbohydrate or other source of energy to serve as a second type of control. As shown in Table 1 the activity resulting from each incubation was expressed in terms of the amount of N.P.N. converted to protein N.

Carbohydrates. The tests made with maize and potato starch (Table 1) showed that when these starches were added to the rumen liquid in the raw state, bacterial activity was much less than with maltose. However, where the solutions of the starches were boiled before use, each produced an amount of bacterial growth considerably greater than that obtained with maltose.

Additions of raffinose, inulin, D-(−)-fructose, and cellobiose each resulted in an amount of protein synthesis of the same order as that brought about by the addition of maltose. This suggests that the rumen bacteria were able to metabolize not only the sugars, fructose, glucose and galactose, but also to break down the types of linkage which exist between the monosaccharide units in raffinose, inulin and cellobiose. The simple sugar, D-(+)-mannose was also as well utilized as maltose, but the bacteria appeared to be unable to use L-(−)-sorbose. This lack of protein synthesis in the presence of sorbose might have been due to its configuration or possibly to a trace of some toxic impurity in the sugar. Sorbose from two different sources was therefore tested. One was a sample obtained commercially, and even after recrystallization it did not result in protein synthesis. The other sample was kindly supplied by Prof. E. L. Hirst, F.R.S., but it also caused no measurable amount of bacterial activity. It would appear probable, therefore, that it was the configuration of the sorbose molecule which rendered it useless to the bacteria.

Of the three pentoses, L-(+)-arabinose and D-(+)-xylose appeared to be well utilized (Table 1), but the remaining one, D-(−)-arabinose, did not result in nearly so much activity as the others. It is interesting to note in this connexion that L-(+)-arabinose is the form of that pentose which occurs most

Table 1. *Protein synthesis in rumen liquid when different substances were added as possible sources of energy for the bacteria*

(Unless otherwise stated, 1% of each substance was added.)

	Control		Experimental	
Type of substance added	Substance added	Protein synthesis (+) or hydrolysis (−) (mg. N/100 g. rumen liquid)	Substance added	Protein synthesis (+) or hydrolysis (−) (mg. N/100 g. rumen liquid)
Carbohydrate	Maltose	+8·9	Raw potato starch	+2·9
			Boiled potato starch	+15·5
			Raw maize starch	+5·5
			Boiled maize starch	+15·8
	Maltose	+9·5	Raffinose	+7·4
			Inulin	+9·2
	Maltose	+13·0	Fructose	+14·0
			Cellobiose	+13·5
	Maltose	+5·7	Mannose	+5·5
			Sorbose	−3·0
	Maltose	+17·6	L-(+)-Arabinose	+21·4
	No addition	−2·0	D-(−)-Arabinose	+3·1
			D-(+)-Xylose	+18·7
Compounds related to hexoses	Maltose	+7·0	Glucuronic acid	−0·6
	Glucose	+7·1	Gluconic acid	−1·0
			Glucosaccharic acid	−0·2
	Maltose	+5·7	Mannitol	+0·6
			Sorbitol	+0·6
	Maltose	+3·6	Alginic acid	+0·9
	Glucose	+7·0	Glucosamine	+0·7
Lower fatty acids	Maltose	+7·2	Sodium acetate	+0·4
	No addition	−1·6	Sodium butyrate	−1·3
	Maltose	+9·6	Sodium propionate	−1·7
	No addition	−3·7		
	Maltose	+9·6	Sodium β-hydroxybutyrate	+3·5
	No addition	+1·6		
Other organic acids	Maltose	+9·2	Lactic acid (0·5%)	−0·5
	Maltose	+14·1	Sodium citrate	−2·7
			Sodium hydrogen malate	−1·2
	No addition	−2·2	Sodium potassium tartrate	−1·3
			Fumaric acid	−0·5
	Maltose (0·3%)	+2·3	Succinic acid (0·3%)	−2·4

commonly in natural products. Since both arabinose and xylose residues may form quite a considerable proportion of the carbohydrate present in vegetable products it is important to know that these pentoses can be utilized so readily by rumen bacteria.

Compounds related to hexoses. The results in Table 1 show that the three oxidation products of glucose, namely glucuronic, gluconic and glucosaccharic acids, when compared with both maltose and glucose, did not enable protein synthesis to predominate. The same was true of the reduction products of fructose and glucose, namely, mannitol and sorbitol, and also of alginic acid and glucosamine.

Lower fatty acids. The results of tests made with these acids are also shown in Table 1. The addition of sodium acetate, propionate or butyrate caused no apparent activity. It would appear, therefore, that these lower fatty acids, which are known to be formed during fermentation in the rumen, are end products and are not readily utilized further by the bacteria.

Other organic acids. The acids tested were β-hydroxybutyric, lactic, citric, malic, tartaric, fumaric and succinic. With the possible exception of β-hydroxybutyric acid none of them appeared to cause any marked bacterial activity. In the test with lactic acid it was found that initially 585 mg. lactic acid per 100 g. rumen liquid were present and that after 4 hr. the value appeared to have decreased to 561 mg., a change which can scarcely be regarded as significant.

A quantitative study of the products formed from maltose, xylose and arabinose by rumen bacteria during in vitro *incubation*

The object of this section of the work was to determine the proportions in which the main end products of carbohydrate fermentation in rumen contents are formed and to find how much of the fermented carbohydrate can be accounted for by the formation of these products.

Incubation technique with gas collection procedure. In a typical experiment 2 l. of rumen liquid to which 1 % of sugar and 0·05 % of urea had been added were divided into two equal portions. One portion, which was contained in a flask fitted with a bung carrying air inlet and outlet tubes, was immediately placed in the incubation bath. The flask was connected to a 10 l. aspirator containing 14 N-H_2SO_4. By slowly emptying this aspirator a CO_2-free air stream was drawn slowly and continuously through the flask to sweep out and collect the gases produced during incubation.

Samples of the other portion were preserved by the addition of H_2SO_4 for the determination of lactic acid and lower fatty acids and by the addition of HCl for the determination of soluble sugars and polysaccharide. A sample preserved with 1 % formalin was also kept for N determinations. To obtain a measure of the total CO_2 including CO_2 of carbonates and bicarbonates present initially in the rumen liquid, a 200 ml. sample of this portion of the unincubated rumen liquid was placed in a flask and connected to gas collection apparatus similar to that described for the incubation flask. After acidification with H_2SO_4 a brisk current of CO_2-free air was drawn through the liquid and approximately 2 l. of the gas mixture collected over 14 N-H_2SO_4. This procedure also gave an estimation of the amount of CH_4 initially present in solution in the rumen liquid.

At the end of the 4 hr. incubation period samples of the incubated rumen liquid were again preserved for analysis. CO_2 and CH_4 remaining in solution were removed as before by acidification followed by brisk aeration, and estimated.

Substances produced during the in vitro *incubation of rumen liquid in the presence of added carbohydrate*

Gaseous products. In each experiment the three gas samples, collected as described, were analysed for carbon dioxide and combustible gas in a Haldane gas analysis apparatus. As a result of combustion the volume of carbon dioxide produced was found to be twice the volume of oxygen used, which suggests in experiments of this type that the combustible gas was mainly, if not entirely, methane. Since, however, the presence of small proportions of other combustible gases such as olefines and carbon monoxide have been reported in gases from the rumen (Olsen, 1942; Dougherty, 1941) samples of gas collected during a typical experiment were treated in an Orsat-Fischer gas analysis apparatus with bromine water to test for olefines and with cuprous chloride to test for carbon monoxide. Olefines and carbon monoxide could not be detected.

In calculating the amount of carbon dioxide which had been produced from the carbohydrate, allowance was made for the carbon dioxide which would theoretically be liberated from added urea by urease activity (Pearson & Smith, 1943*b*) and also from carbon dioxide liberated from carbonates and bicarbonates as a result of acid production during incubation. Methane figures were also corrected for any methane originally present in solution at the beginning of incubation and removed by aeration during the incubation period. The amounts of both gases formed in experiments with maltose, xylose and arabinose are recorded in Table 2. In presenting the results the fermentation products have been expressed in terms of the carbon they contained so that the amounts formed can be compared on a common basis with the amount of carbohydrate fermented.

Table 2. *Amount of carbohydrate expressed in terms of carbon which was utilized during incubation and which was accounted for by the carbon of the various end products formed*

(Three experiments were made with maltose, one with arabinose and two with xylose.)

	Amount formed during incubation (mg. C/100 g. rumen liquid)						Percentage of carbohydrate C accounted for by each product					
Added carbohydrate ...	Maltose			Arabinose	Xylose		Maltose			Arabinose	Xylose	
Exp.	1	2	3		1	2	1	2	3		1	2
End products												
Volatile fatty acids	88*	44*	104*	62*	77	59	35·8	23·6	44·8	41·3	52·4	44·5
Lactic acid	14	18	20	1	0	0	5·7	9·6	8·6	0·7	0·0	0·0
Carbon dioxide	18	13	22	6	12	2	7·3	7·0	9·5	4·0	8·2	1·5
Methane	8	3	10	2	4	2	3·3	1·6	4·3	1·3	2·7	1·5
Bacterial protein	16	28	32	25	21	24	6·5	15·0	13·8	16·7	14·3	18·0
Bacterial polysaccharide	76	72	34	28	22	24	30·9	38·5	14·7	18·7	15·0	18·0
Total	220	178	222	124	136	111	89·5	95·3	95·7	82·7	92·6	83·5
Carbon of carbohydrate changed during incubation	246	187	232	150	147	133						

* These figures were obtained by estimating total acidity and applying the factor derived in Table 3.

Volatile fatty acids. The results for fatty acids given for four of the experiments in Table 2 are marked with an asterisk to denote that only total volatile acidity was determined and that the figures shown were calculated by using a factor obtained from later experiments in which the acids were partitioned into acetic, propionic and butyric fractions. The results of these later experiments are shown in Table 3, which gives details of fatty acid production in tests in which the silica-gel partition chromatogram technique was employed. Some of the tests were made with rumen contents obtained from animals at pasture, and some with rumen contents from animals which were fed indoors on hay, oats, dried grass and beans. The results show that with a grass diet the rumen contents tended to produce acetic and propionic acid in equimolecular proportions, whereas with rumen contents obtained when the animals were stall-fed, the molecular ratio of acetic acid to propionic acid was considerably greater than 1. Butyric acid was produced with some samples of rumen contents, but with others no measurable amount could be detected. This difference between samples was not related to the type of diet fed to the animals nor did it depend on whether the sugar being utilized was maltose, arabinose or xylose. For the present work, the most important fact arising from Table 3 is that the carbon content of the acids produced (expressed as mg. C/ml. 0·1N-acid) varied only within relatively narrow limits and averaged 3·08. Where, therefore, a factor was used to obtain the figures in Table 2, this value was adopted.

Lactic acid. Lactic acid production figures are given in Table 2. It is in these figures that the most striking difference existed between the results with maltose and those for the two pentoses. From 5 to 10% of the fermented maltose was recovered as lactic acid, whereas less than 1% of the arabinose and none of the xylose could be accounted for by formation of this acid.

Bacterial protein. In calculating the amount of carbon in bacterial protein, the assumptions were made that all the nitrogen of the sediment isolated was protein and that the nitrogen content of bacterial protein is 16% and the carbon content 53%. Table 2 shows that from 6·5 to 18% of the carbon of the fermented sugar was recovered as bacterial protein.

Bacterial polysaccharide. Since the bacterial sediment gave a strongly positive reaction when tested for starch with iodine the bacterial polysaccharide was calculated as starch. In two of the maltose experiments approximately one-third of the fermented maltose carbon was recovered as polysaccharide, whereas in the other four experiments only about one-sixth was recovered in that form. In an early experiment with pentose, the staining reaction did not indicate the presence of starch, suggesting that possibly a pentosan was being stored. In the present experiments, however, tests for pentose in the bacterial sediment showed that sediments from hexose and pentose incubations contained equal amounts of pentose (4·1% in one experiment in which maltose and arabinose were compared), indicating that there had been no greater deposition of pentosan by the organisms which were utilizing pentose.

Table 3. *Composition of fatty acid mixtures produced during* in vitro *incubation of rumen liquid with maltose, arabinose and xylose*

Diet of animal from which rumen liquid was obtained	Exp. no.	Carbohydrate added	Fatty acid production during incubation (ml. 0·1N-acid/100 g. rumen liquid)				Carbon content of acids produced		
			Acetic	Propionic	Butyric	Total	(mg. C/100 g. rumen liquid)	(mg. C/ml. 0·1N-acid)	Ratio, Acetic/propionic
Pasture grass	1	Maltose	10·5	10·5	—	21·0	63·0	3·00	1·00
	2	Maltose	11·6	12·0	1·8	25·4	79·7	3·14	0·97
		Arabinose	12·6	14·2	1·5	28·3	88·6	3·13	0·89
Indoor diet of concentrates and hay	3	Maltose	9·3	5·0	4·2	18·5	60·5	3·27	1·86
		Xylose	12·3	9·2	3·1	24·6	77·5	3·15	1·34
	4	Maltose	10·0	6·7	0·7	17·4	51·5	2·96	1·49
		Xylose	12·3	8·6	—	20·9	60·5	2·90	1·43
							Mean	3·08	

The changes undergone by various rumen liquid constituents during in vitro *incubation in the absence of added carbohydrate*

In calculating the recovery of added carbohydrate as volatile fatty acid, etc., in Table 2 the assumption was made that the various fermentation products estimated arose from the added carbohydrate. The possibility that some of these might be formed in significant amounts from other constituents of the rumen liquid even during an incubation period of 4 hr. had also to be considered. To investigate this point two separate experiments were carried out in which rumen liquid was incubated under conditions

similar to those already described except that no carbohydrate was added. Under these circumstances very little change could be detected. Protein was the main constituent to be affected, but the amount decomposed in 4 hr. was equivalent to only about 9 mg. carbon/100 g. rumen liquid, whereas when 1 % sugar was present, the change which occurred in the same time was equivalent to as much as 133–246 mg. carbon/100 ml. (Table 2).

DISCUSSION

The first section of the work has confirmed that if in 4 hr. incubations the vigorous bacterial growth observed by Pearson & Smith (1943*a*) and by Smith & Baker (1944) is to occur, the presence of dissolved starch or sugar is essential. From the present work and that of Pearson & Smith (1943*a*) it is clear that in addition to starch, the bacteria are able to use the polysaccharide inulin, the trisaccharide raffinose, the disaccharides maltose, lactose, cellobiose and sucrose, the hexoses D-(+)-galactose, D-(+)-mannose and D-(−)-fructose, and the pentoses L-(+)-arabinose and D-(+)-xylose. They were unable to use L-(−)-sorbose and showed only limited activity with D-(−)-arabinose. Apparently, therefore, they can readily utilize many different carbohydrates, but it is clear that with some sugars the configuration of the molecule may inhibit the ability of the bacteria to ferment them. The necessity for the presence of a potential reducing group and a primary alcohol group in the molecule if it is to be utilized by the bacteria was made clear by the fact that oxidation products of glucose such as gluconic, glucuronic and glucosaccharic acids, and a reduction product such as mannitol did not result in marked protein synthesis.

In addition to sugars and related compounds, volatile fatty acids were tested. It is well established that they are products of fermentation in the rumen and the tests were made to determine whether they were further decomposed by rumen bacteria in the absence of added carbohydrate. The results indicated that in 4 hr. incubations they were not utilized. These observations agree with the conclusions of Phillipson & McAnally (1942), who found that the volatile fatty acids were stable in rumen contents for at least 7·5 hr. In the same experiments Phillipson & McAnally found that lactic acid disappeared rapidly from the rumen, but that when rumen contents were filtered through muslin, they had a variable effect upon lactate *in vitro*, and that the supernatant liquid obtained by centrifuging rumen contents had no effect. Elsden (1945–6) found that during prolonged *in vitro* incubation of diluted rumen contents with glucose, lactic acid accumulated during the first stage of fermentation and was later changed to volatile fatty acid. In the present work lactic acid did not appear to be changed to any significant extent when incubated with fresh rumen contents for 4 hr. Several other acids such as citric, tartaric and succinic were tested, but they did not induce any vigorous bacterial growth as measured by protein synthesis.

The second part of the work was designed to study the quantitative aspect of carbohydrate breakdown in rumen contents. It was found that in three experiments with maltose 90, 95 and 96 % of the carbon of the fermented sugar could be accounted for by the formation of acetic, propionic, butyric and lactic acids, carbon dioxide, methane and bacterial polysaccharide and protein. With xylose the corresponding figures were 84 and 93 %, and in the one experiment with arabinose the value was 83 %. The fact that 4–10 % was unaccounted for with maltose, and as much as 17 % with the pentoses, may be attributed to two causes. In the first place the determinations of the several end products are subject to errors, some of which may well be cumulative. For example, Heald (1951) has shown that not all of the reducing substances obtained on hydrolysing rumen micro-organisms are fermentable sugars. Consequently the use of reduction methods for estimating sugars in complex work of this type cannot be other than approximate. Heald found, however, that the amount of non-fermentable reducing substances in the rumen contents of sheep remained fairly constant throughout the digestive cycle. It would seem, therefore, that by measuring changes in the total reducing substances a fair estimate of the changes in fermentable material would be obtained.

In the second place, only the main products of the bacterial activity were determined. Marston (1948), who described somewhat similar experiments on the fermentation of cellulose in rumen contents, in which he obtained recoveries of about 96 %, tested for intermediate products such as succinic acid, acetaldehyde, other aldehydes and ketones, lactic acid and pyruvic acid, and concluded that they did not appear in quantities sufficient to affect the recovery figures. Of these substances only lactic acid was tested for in the present work. It was formed in significant amounts from maltose, but not from the two pentoses. Since the recoveries with the pentoses tended to be less (83–93 %) than those obtained with maltose (90–96 %), it may well be that when pentoses are decomposed in rumen contents some compound, not estimated in the present studies, is formed instead of the lactic acid which is formed from maltose. This point requires further investigation.

In Marston's experiments cellulose was used as the source of carbohydrate and incubations were necessarily much longer than in the present work. The technique also differed in that the micro-organisms were first isolated from rumen contents,

washed and suspended in an inorganic medium containing cellulose. Marston's results differed from the present findings in several aspects. For example, the average amount of cellulose carbon recovered as lower fatty acids was 73 % and as gaseous products 21 %, very little being accounted for by the formation of bacterial protein and polysaccharide. In the 4 hr. sugar incubations of the present work, a very much higher proportion of the recovered carbon was present in the bacteria. This observation agrees with the theory that maximal bacterial proliferation and utilization of N.P.N. occurs during the short period of vigorous fermentation which follows a feed, when readily available carbohydrate is rapidly broken down. In this connexion it has been shown in feeding experiments by Rupel, Bohstedt & Hart (1943) and by Willett, Henke & Maruyama (1946) that maximum N.P.N. utilization occurs when the diet is rich in carbohydrates which the bacteria can utilize readily and rapidly.

Very little information is available in the literature regarding the amounts of the various products which are formed from simple sugars in the rumen of intact animals, but Phillipson & McAnally (1942) observed that when 100 g. galactose, a sugar which they found behaved similarly to maltose in the rumen, was given to a sheep, the increase in volatile fatty acids calculated as acetic acid in the rumen liquid amounted to 170 mg./100 ml. within 4·5 hr. The carbon content of this amount of acetic acid is 68 mg. and is within the range of volatile fatty acid production (44–88 mg. C/100 g. rumen liquid) found in the present investigations (Table 2).

The figures for the production of carbon dioxide and methane in the present work varied considerably from one experiment to another, and the ratio of carbon dioxide to methane formed varied from 1·0 to 4·3. The average of these ratios, however, was 2·63, a figure which agrees well with that of 2·58 reported by Kleiber, Cole & Mead (1943) for the rumen gases of intact animals. The present figure is similar also to that of 2·40 obtained by Washburn & Brody (1937) for the gas from the rumen of a cow slaughtered 4 hr. after a feed.

These considerations lead to the conclusion that in the *in vitro* experiments of Pearson & Smith (1943*a*, *b*, *c*) and Smith & Baker (1944) the changes which occurred when rumen liquid was incubated with soluble carbohydrate were very similar to those occurring in the rumen itself, particularly in the few hours following a meal. It seems probable, therefore, that conclusions regarding protein synthesis and breakdown drawn from results of the *in vitro* work described in earlier parts of the present series may reasonably be considered to apply also to the intact animal.

SUMMARY

1. A study has been made of the ability of several carbohydrates and other compounds to support vigorous bacterial growth in bovine rumen contents. Each substance was incubated in fresh rumen liquid at a concentration of 1 %. Portions of the same rumen liquid were incubated with 1 % maltose or glucose as control.

2. The polysaccharides starch and inulin, the trisaccharide raffinose and several disaccharides, hexoses and pentoses were found to be readily utilized by the bacteria. Since oxidation products of glucose such as gluconic and glucuronic acids and a reduction product such as mannitol produced no measurable activity, it would appear that both a potential aldehyde and a primary alcohol group are essential. Molecular configuration was also important since fructose was readily fermented while sorbose showed no activity. Similarly, L-(+)-arabinose gave marked bacterial growth, whereas with D-(−)-arabinose activity was much more limited.

3. The other substances tested included lower fatty acids, lactic acid, succinic and tartaric acids. None of them gave rise to bacterial growth as measured by protein synthesis.

4. A quantitative study has been made of the principal products formed when maltose, arabinose and xylose are utilized *in vitro* by the microflora of the bovine rumen during an incubation period of 4 hr.

5. In three experiments with maltose 90–96 % of the carbon of the maltose fermented was accounted for by the formation of lower fatty acids, lactic acid, carbon dioxide, methane, bacterial protein and polysaccharide.

6. In two experiments with xylose and one with arabinose 83–93 % of the carbon of the decomposed sugar was accounted for.

7. The results for the pentoses differed from those obtained with maltose in that with the pentoses no lactic acid was detected among the end products.

The author wishes to thank Dr J. A. B. Smith for his continued interest and advice while the work was in progress. She is also most grateful to Prof. E. L. Hirst, F.R.S., Prof. S. Peat, F.R.S., and Prof. M. Stacey, F.R.S., for samples of carbohydrate derivatives, to Dr P. S. Watts, F.R.C.V.S., for his help in looking after the fistula animals, and to Messrs Imperial Chemical Industries Ltd. for a grant towards the cost of the work.

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Ranunculin: The Precursor of the Vesicant Substance of the Buttercup

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Many of the plants belonging to the natural order Ranunculaceae have the property of producing erythema and blistering the skin. From these plants a terpene-like substance can be obtained, which has long been known under the name of anemonin (I). Anemonin, however, has no vesicant properties, and is derived from the active substance by polymerization. The constitution of the active substance known as protoanemonin (II) has been elucidated by Yasuhiko & Fujita (1922) and Kipping (1935). This was shown to be the lactone of γ-hydroxyvinylacrylic acid (III) and is an oil, volatile in steam and very soluble in water. The pure substance or its solution in water polymerizes in the course of a few days to the insoluble crystalline product anemonin. The distribution of protoanemonin among a series of *Ranunculus* spp. was examined quantitatively by Shearer (1938).

The present investigation was undertaken with the object of finding how the protoanemonin was both stable and harmless within the plant itself. A further object was to obtain a readily available supply of protoanemonin for examination of its action on enzymes, so that the properties of this very simple natural vesicant could be compared with those of the well known vesicants.

It has been found that when the tissues are crushed the protoanemonin is liberated enzymically from a glucosidic precursor in the plant. The glucoside itself has been isolated as a crystalline substance very soluble in water, and, unlike protoanemonin itself, is stable both as a solid and in aqueous solution. This substance, for which structure (IV) is suggested, we propose to call ranunculin, as it has been obtained from several species of *Ranunculus*. Ranunculin, while very stable in acid solution, is rapidly broken down in alkaline solution with the liberation of glucose. The protoanemonin thus formed is converted into acetylacrylic acid (V). Ranunculin, on distillation with aqueous sodium acetate, gives a nearly quantitative yield of protoanemonin and is thus a convenient stable source of the latter.

The ease of production of protoanemonin (II) suggests that in the glucoside the sugar residue is attached to a substance closely related to the lactone. Protoanemonin itself could not be regarded, however, as the aglucone because it has no free hydroxyl group. The most natural assumption would be that the glycoside is that of the enol form of acetylacrylic acid. However, as neither the glycoside nor the tetra-acetyl derivative shows the presence of a free carboxyl group, this assumption could not be true. The rapid liberation of the glucose by alkali and the relative stability of the glucoside in acid solution would point to its being a glucose ester of acetylacrylic acid. This, however, will not easily explain the ready formation of the lactone by boiling with sodium acetate solution or as the initial product of

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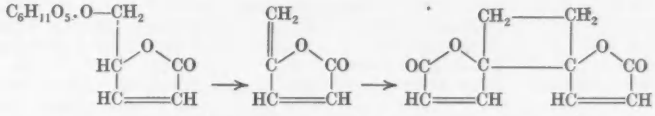
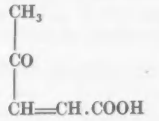
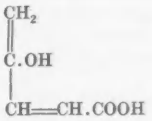
dilute alkali treatment in the cold. If it is assumed that the lactone ring already exists in the glucoside then a structure of the type (IV) results. This hypothetical structure explains the properties so far observed. It is seen that if we adopt this structure liberation of the anhydroaglucone, protoanemonin, will occur without the addition of water, which is an unusual case of the breakdown of a glycoside being analogous to the glycoside picrocrocin described by Kuhn & Winterstein (1933). The properties of the glucoside are such that it would accompany sugars in the usual methods of extracting plant tissues for estimating carbohydrates. Further, it would break down to give glucose during a method of copper reduction. As, in some cases, the substance is present as 1 % of the fresh weight of the plant, it would make it appear that the plant had a high level of hexose, unless the presence of this type of glucoside was suspected.



Ranunculin (IV) (provisional structure) Protoanemonin (II) Anemonin (I)



β-Acetylacrylic acid (V)



γ-Hydroxyvinylacrylic acid (III)

EXPERIMENTAL

The formation of protoanemonin from the plant

The fresh plant of *Ranunculus bulbosus* on being bruised in a mortar develops a strong smell of protoanemonin in the course of 3–5 min.; the effect is not instantaneous. The fresh plant bruised with dilute acid in a mortar (pH 2, to destroy all enzymes) does not develop protoanemonin. If the fluid extract is now removed and boiled with sodium acetate, protoanemonin is volatilized with the steam.

It is therefore concluded that protoanemonin is liberated by enzyme action from a precursor in the plant, and that the precursor is decomposed by heating with sodium acetate.

Isolation of the precursor from the plant

The fresh plants were ground in lots of 180 g. with 200 ml. water containing either 5 ml. conc. HNO_3, or 8 ml. conc. HCl. The pulp was pressed out through a cloth and the fluid spun on a centrifuge. The brownish turbid fluid was then filtered through kieselguhr to remove suspended matter. The clear brownish filtrate was then treated with charcoal (3–6 g./180 g. fresh plant) for 20 min. at room temperature. The charcoal was British Drug Houses 'activated', previously boiled in a small quantity of distilled water to expel the air. After this treatment the solution was pale straw-yellow.

The nearly colourless fluid was then treated in a similar way with a larger quantity (up to 20 g./180 g. plant) of charcoal, which now adsorbed the precursor. The charcoal was filtered off on large Büchner funnels on papers covered with a thick layer of kieselguhr. The charcoal was carefully washed with distilled water to remove excess of acid and unadsorbed matter, and the precursor was eluted from the charcoal by slowly sucking 50 % aqueous ethanol through it.

The filtrate was evaporated to a thin syrup under reduced pressure, washed out of the vessel with a small quantity of water and treated with 2 vol. of methanol. A precipitate containing pectin was filtered off and the clear filtrate evaporated down *in vacuo* and finally taken down (in a dish) to a thick syrup, in an air current on a warm bath. Towards the end of the operation the syrup was seeded and stirred until it became a semi-solid mass of crystals. After cooling, small quantities of methanol were added with thorough mixing, until after 20 min. the substance had all crystallized out. It was then filtered off and washed with methanol. A colourless, nearly pure, preparation was thus obtained. Up to 0·9 g. was obtained for each 10 g. charcoal used. Recrystallization was effected by dissolving in warm methanol (12 parts), filtering, and treating as in the isolation of the crude substance.

The melting points of the crude products were usually in the neighbourhood of 140°; in a few cases the products sintered at 135°. The substance was obtained from various species over a period in the early summer of 1942. The identity of the substance from the different species was established by seeding and by mixed melting points. The results of the various preparations are shown in Tables 1 and 2. The yields of ranunculin which we obtained correspond with the protoanemonin found by Shearer (1938) from the same species.

Properties of ranunculin

The substance is a colourless solid crystallizing in nearly rectangular plates, sometimes much elongated, of which 1 g. dissolves in 0·7 ml. of water. Large crystals are formed on evaporation of such a solution. It is sparingly soluble in cold

Table 1. *Isolation of glucoside from four* Ranunculus *species*

	Plant		Yield	
Date, 1942	Species	(g.)	(g.)	Percentage of fresh wt. of plant
18 May	*R. bulbosus*	180	0·6	0·33
19 May	*R. bulbosus*	335	3·25	0·97
26 May	*R. bulbosus*	610	5·37	0·88
30 May	*R. bulbosus*	680	5·45	0·80
27 May	*R. acris*	540	1·05	0·19
8 June	*R. acris*	1300	4·70	0·36
8 June	*R. acris*	800	3·42	0·40
3 June	*R. arvensis*	1050	7·7	0·58
16 June	*R. arvensis*	1260	20·55	1·63
24 June	*R. arvensis*	1080	8·4	0·78
6 July	*R. sceleratus*	500	3·05	0·61

Table 2. *Efficiency of charcoal adsorption in the preparation of ranunculin*

Date, 1942	Species	Charcoal used for nearly complete adsorption (g.)	Yield (g.)	M.p. of crude product (°)
26 May	*R. bulbosus*	50	4·52	139–140
30 May	*R. bulbosus*	50	4·35	139
16 June	*R. arvensis*	100	8·70	136–138

methanol and nearly insoluble in cold ethanol. No loss of weight occurs at 100° *in vacuo* over P_2O_5. The *substance* melts at 141–142° giving a colourless liquid; the melting point is markedly depressed by traces of water. Visible decomposition occurs in the melting point tube only at about 205°. (Found: C, 48·1; H, 5·9. $C_{11}H_{16}O_8$ requires C, 47·8; H, 5·8%.) The substance is laevorotatory $[\alpha]_D^{17.5°} = -80·7°$ (0·417 g. in 25 ml. water, $l = 20$ cm., $\alpha = -4·69°$).

Reactions of ranunculin

Action of alkali. If dilute NaOH is added to a concentrated solution of ranunculin the smell of protoanemonin is noted, but this soon becomes inappreciable. The decomposition with alkali was examined polarimetrically both with NaOH and Na_2CO_3. In both cases at room temperature the rotation changed rapidly towards that of a glucose solution treated similarly (Tables 3 and 4). During the reaction with carbonate an amorphous precipitate formed, soluble in alkali, indicating that side reactions occur.

Table 3. *Action of* NaOH *on ranunculin at* 19°

(0·714 g. ranunculin in 25 ml. 0·1 N-NaOH. 0·500 g. glucose in 25 ml. 0·1 N-NaOH. 0·714 g. ranunculin, $C_{11}H_{16}O_8$, contains 0·5 g. glucose.)

Value of α	Ranunculin	Glucose
Initial	−4·69°	+2·10°
10 min.	+1·15°	+1·83°
1 hr.	+1·19°	+1·60°
24 hr.	+1·02°	+0·47°

Table 4. *Action of* K_2CO_3 *on ranunculin at* 19°

(0·714 g. ranunculin in 25 ml. water + 0·2 g. K_2CO_3. 0·500 g. glucose in 25 ml. water + 0·2 g. K_2CO_3.)

Value of α	Ranunculin	Glucose
Initial	−4·69°	+2·1°
10 min.	−0·80°	+2·1°
24 hr.	+1·42°	+2·1°

Titration with alkali. The glucoside was titrated with alkali at 60° using phenolphthalein or alizarin as indicator. The result was in agreement with there being one lactone ring per molecule of glucose in the compound (Table 5).

Table 5. *Titration of glucoside*

	0·1 N-NaOH (ml.)
Calc. for 0·200 g. substance $C_{11}H_{16}O_8$ (mol.wt. 285)	7·20
0·199 g., indicator phenolphthalein, required	7·07
0·199 g., indicator alizarin, required	7·35

Estimation of free COOH *groups.* Ranunculin displaced no CO_2 from bicarbonate buffer, pH 7·4, 37°, in Barcroft manometers. It was therefore concluded that the COOH group is combined as a lactone ring.

Acetyl derivative. Ranunculin (0·5 g.) was well broken up in a mixture of 12 ml. acetic anhydride and 1·0 ml. glacial acetic acid; 10 ml. pure pyridine were added very slowly with cooling. After 2·5 hr. the mixture was poured on to ice, and the pyridine and part of the acetic acid were distilled off *in vacuo*. The crude substance was precipitated by water and the insoluble residue was crystallized first from absolute ethanol and then from very dilute aqueous methanol.

The *acetyl* compound crystallizes in long colourless needles, m.p. 136–137°. It is appreciably soluble in hot water. (Found: C, 51·4; H, 5·4. $C_{19}H_{24}O_{12}$ requires C, 51·4; H, 5·4%.)

Titration of acetyl compound. (*a*) 0·1 g. in 20 ml. water was titrated at 60° with 0·1 N-NaOH, using phenolphthalein as indicator. The fluid became yellow during the titration. (Found: 11·4 ml. Calc. for 4 acetyl and 1 lactone group: 11·3 ml.) (*b*) 0·102 g. in 12 ml. 0·1 M-NaOH was kept at 60°

for 20 min. and then back titrated with 0·104 N-HCl, using phenolphthalein. (Found: 11·82 ml. 0·1 N-NaOH used. Calc. 11·45 ml.)

Estimation of glucose. The glucoside is so rapidly decomposed by alkali that it reduces Fehling's solution like free glucose. It was titrated with Fehling's solution by the method of Lane & Enyon (1923). (Found 63% glucose. Calc. (structure IV) 70%.)

Formation of glucosazone. Ranunculin was boiled with dilute sodium acetate for 5 min. and then treated at 100° for 20 min. with phenylhydrazine and dilute acetic acid. Glucosazone was produced. No glucosazone was given under similar conditions from the glucoside itself, without previous hydrolysis.

Formation of protoanemonin. The glucoside (0·2 g.) was dissolved in 5 ml. water containing 2 g. sodium acetate, and the solution was steam distilled for 25 min. The distillate was then neutralized using phenolphthalein (1·6 ml. 0·1 N-NaOH were required to neutralize free volatile acid). 8 ml. 0·1 N-NaOH were added and the solution kept at 60° for 45 min. Back titration with 0·1 N-HCl showed that 5·9 ml. 0·1 N-NaOH were used.

The distillation was continued for a further 30 min. and the distillate neutralized as before. Back titration: 0·55 ml. Total NaOH: 6·45 ml. Calc. for structure IV: 7·2 ml. 0·1 N-NaOH.

Preparation of protoanemonin. Ranunculin (3 g.) was dissolved in 300 ml. 10% sodium acetate. This solution was distilled at atmospheric pressure for 25–30 min., or until no more protoanemonin came over. Any acetic acid in the distillate (about 150 ml.) was neutralized with $NaHCO_3$, and the solution was saturated with NaCl and extracted eight times with ether. The ether extract (about 120 ml.) was shaken with a little more $NaHCO_3$, dried over Na_2SO_4, and the ether removed *in vacuo*. The residue was a liquid having the vesicant properties described for protoanemonin; it very rapidly polymerized to a solid.

SUMMARY

1. The protoanemonin obtained from species of buttercups (*Ranunculus*) is derived from a glucoside.

2. The isolation and some properties of the glucoside are described; a provisional structure is given.

3. The substance seems likely to be present only in Ranunculaceae, and the name ranunculin is proposed for this glucoside which yields protoanemonin.

4. The pure glucoside is a stable preparation which is a convenient source of protoanemonin.

This work formed part of a programme of research carried out for the Chemical Defence Development Department of the Ministry of Supply by an extramural research team under the direction of Dr M. Dixon, F.R.S., and was reported to the Ministry (Hill & van Heyningen, 1943). We are indebted to the Director General of Scientific Research (Defence) of the Ministry of Supply for permission to publish this work. We would like to thank Dr B. Lythgoe for his suggestion that ranunculin shows analogy with crocetin and for his help in stating the most probable provisional constitution for the former.

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Suppression of Catalase Activity by Peroxidase and its Substrates

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While studying the darkening of doughs made from low-grade flours, the authors wished to ascertain whether the peroxidase of wheat germ could function in the presence of the catalase also present in germ. A vast amount of work has been done on catalase as well as on peroxidase, but little seems to be known of what happens on addition of hydrogen peroxide to a system showing both catalase and peroxidase activities. Zeile (1934) expresses the opinion that peroxidase activity is inhibited in the presence of catalase. Later in the same article he says that, if catalase is present in small amounts only, peroxidase activity is predominant, whereas large amounts of catalase cause purely oxidative effects by providing molecular oxygen for oxidases. This is how Sumner & Somers (1947) sum up the situation: 'It would appear that peroxidase is of value to plants and animals to bring about the oxidation of certain phenols, but there is little or no experimental evidence to support this. Whether enough hydrogen

peroxide can occur in cells which contain catalase is dubious; whether sufficient phenols are likely to be present is also somewhat dubious. Finally, we can see no especial value to the animal in the formation of such products of oxidation as quinone, for example. It is possible that peroxidase serves a purpose by removing hydrogen peroxide, but here again we have no evidence.'

A preliminary qualitative test showed that a wheat-germ suspension, on addition of hydrogen peroxide, gave a strong catalase reaction. When a small quantity of a peroxidase substrate, e.g. catechol, was added, the oxygen liberation stopped, darkening began immediately and proceeded to a maximum, after which oxygen liberation resumed. Alternating additions of hydrogen peroxide and substrate resulted in alternating induction and suppression of oxygen evolution, suppression being accompanied by oxidation of the substrate. As a result of this observation a series of quantitative experiments was carried out. The results were considered of sufficient general interest to warrant extending the study to other materials, including an artificial catalase-peroxidase system of purified horse-liver catalase and horse-radish extract. The results have a bearing on certain observations made by other workers and on the accepted methods of catalase determination.

EXPERIMENTAL

Materials

Suspensions of wheat germ (Kat.f. 0·44, P.Z. 0·4) and horse-radish (P.Z. 2·92) were used as sources of peroxidase and catalase. Purified catalase (Kat.f. 7670) was prepared from horse liver by the method of Keilin & Hartree (1945*a*). For the definition of catalase activity (Kat.f.) and peroxidase activity (P.Z.) see Euler & Josephson (1927) and Willstätter & Pollinger (1923) respectively. Catechol, quinol, pyrogallol, phloroglucinol and *p*-phenylenediamine were used as substrates. The H_2O_2 solution was made by diluting a commercial 130 vol. preparation.

Methods

The quantitative method used was based on the following considerations. If a suspension or solution showing catalase and peroxidase activity receives successive additions of hydrogen peroxide, each of them small enough to be completely decomposed by the catalase within a short time, say 5 min., then the volumes of oxygen recorded after each addition should depend only on the quantity of hydrogen peroxide added. If peroxidase, in the presence of a substrate, is able to compete with catalase for the limited amount of hydrogen peroxide added, then the volume of oxygen evolved after each addition of hydrogen peroxide should be reduced. These conditions were taken to be similar to those existing in the living cell which is assumed to decompose small amounts of hydrogen peroxide rapidly and completely.

The apparatus used in carrying out the experiments consisted of a 200 ml. wide-necked conical flask fitted with a rubber stopper carrying the tip of a microburette and a capillary-tube T-piece opening via a glass stopcock on one side to the air and connected on the other side to a U-tube water manometer fitted with a graduated tube and levelling bulb for volume measurement. The other side of the manometer was connected by means of a similar T-piece to a 200 ml. temperature-compensating flask. Both flasks were immersed in a water bath maintained at 26·5° and the whole assembly was shaken in a horizontal plane at the rate of 180 oscillations per min. (amplitude 40 mm.).

The catalase-peroxidase enzyme material, suspended or dissolved in 20 ml. 0·2 M-phosphate buffer, pH 6·8, was placed in the reaction flask. When temperature equilibrium had been reached, 0·2 ml. of 0·64 N-H_2O_2 was added from the microburette and shaking commenced. After 5 min. the volume of liberated O_2 at atmospheric pressure was measured. The initial condition of the system was then restored by expulsion of this gas and the procedure repeated by adding a further 0·2 ml. H_2O_2. Several such additions were made and O_2 volumes taken to establish that part of the graph which is due to total decomposition of H_2O_2 by catalase.

Peroxidase substrate, usually in an amount to give a 0·01 M solution in the reaction mixture, was then added and when temperature equilibrium had been restored the stepwise addition of H_2O_2 was continued.

RESULTS

In all experiments, whatever the source of catalase and peroxidase, the addition of one of the familiar substrates of peroxidase resulted in oxidation of the substrate and severe suppression of oxygen liberation. Fig. 1 shows the typical effect on the catalase

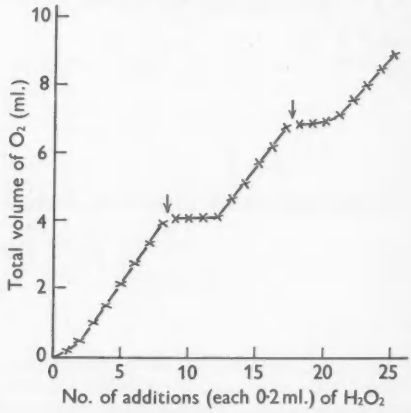

Fig. 1. Effect of quinol on the catalase activity of wheat-germ suspensions. Arrows indicate addition of 22 mg. quinol to suspension of 2 g. finely divided wheat germ (Kat.f. 0·44, P.Z. 0·4) in 20 ml. 0·2 M-phosphate, pH 6·8.

activity when two successive additions of quinol were made to a wheat-germ suspension. After the bulk of the substrate had been oxidized the volumes of oxygen set free for successive additions of hydrogen peroxide began to increase, eventually reaching the level found before addition of the substrate. A second addition of substrate had the same effect in suppressing catalase activity. When the amount of substrate was increased the flat portion of the graph, representing suppression of catalase activity, was lengthened proportionately, but when the substrate concentration in the reaction mixture exceeded a certain limit, for example 0·02 M, the final slope of the graph did not quite revert to the initial angle.

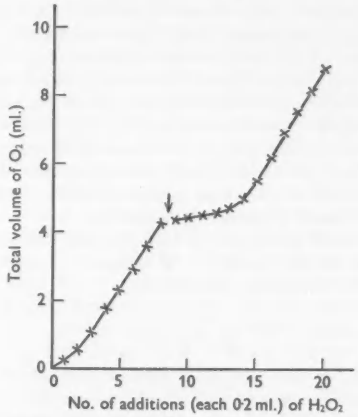

Fig. 2. Effect of *p*-phenylenediamine on the catalase activity of wheat-germ suspensions. Arrow indicates addition of 21·6 mg. *p*-phenylenediamine to suspension of 2 g. finely divided wheat germ (Kat.f. 0·44, P.Z. 0·4) in 20 ml. 0·2 M-phosphate, pH 6·8.

With other substrates the trend was the same, although the return to the initial slope was somewhat more gradual. This is shown in Fig. 2, which represents the effect of *p*-phenylenediamine.

Experiments showed that the initial slight bend seen in the graphs (Figs. 1 and 2) was due mainly to a substrate naturally occurring in wheat germ; the presence of reducing matter such as glutathione (GSH) was a minor contributory factor. Randall's (1946) finding that thiol compounds act as peroxidase substrates was confirmed, but the amount of GSH in the germ suspension used was insufficient to account for more than a small part of this initial suppression of oxygen liberation. The addition of hydrogen peroxide to a germ suspension was accompanied by a colour change from yellow to pink, probably due to oxidation of the natural substrate.

The ratio of catalase to peroxidase was varied by addition of horse-liver catalase to germ suspensions. With such systems it was found that suppression of oxygen liberation depended not only on the ratio of catalase to peroxidase activities, but also on the absolute concentration of the enzymes. Suppression of oxygen liberation was negligible in a system containing 0·086 mg. of horse-liver catalase and 2000 mg. of wheat germ, but was almost complete in an experiment where 100 mg. of germ were used (Fig. 3). The divergence of the initial part of the curves is due to the different concentrations of natural substrates.

An artificial system containing 0·086 mg. of horse-liver catalase (Kat.f. 7670) and 57·4 mg. of horse-radish (P.Z. 2·92) was also used to study the effect of a catalase:peroxidase ratio different from that of wheat germ. Fig. 4 illustrates the behaviour of this system when quinol was added.

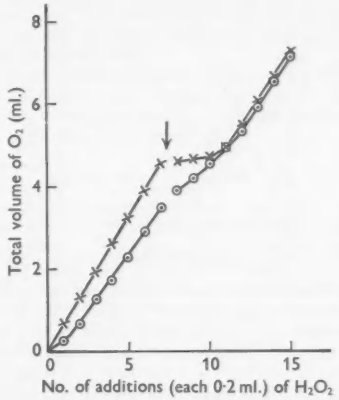

Fig. 3. Effect of quinol on systems containing 0·086 mg. horse-liver catalase (Kat.f. 7670) and 2000 mg. (⊙—⊙) or 100 mg. (×—×) of wheat germ (Kat.f. 0·44, P.Z. 0·4) suspended in 20 ml. 0·2 M-phosphate, pH 6·8. Arrow indicates addition of 22 mg. quinol.

Fig. 3 shows that the suppression of catalase activity in the artificial system was definite though somewhat less marked than in wheat-germ suspensions. The other substrates produced similar suppressions of catalase activity, although, as with wheat-germ suspensions, the return to the initial slope was more gradual.

Keilin & Hartree (1945*b*) found that catalase is able to oxidize certain alcohols by using the oxygen evolved in the decomposition of hydrogen peroxide, and suggested that it might be able to oxidize other biologically important compounds in the same way.

In order to make sure that the observations reported above were not due to purely catalatic oxidation, the authors used the method described in this paper in an attempt to oxidize typical peroxidase substrates by means of purified catalase. The degree of oxidation did not exceed that obtained in a control

experiment where catalase was absent, but the volume of oxygen evolved in the usual 5 min. reaction time showed that the peroxidase substrates listed under the Materials section, when used in concentrations of 0·01–0·02 M at pH 6·8, caused severe reduction of catalase activity. This decrease in

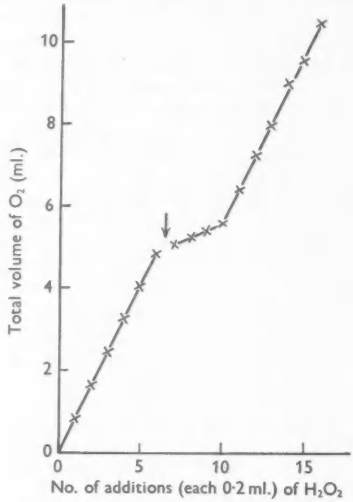

Fig. 4. Effect of quinol on a system containing 0·086 mg. purified horse-liver catalase (Kat.f. 7670) and 57·4 mg. horse-radish extract (P.Z. 2·92) in 20 ml. 0·2 M-phosphate, pH 6·8. Arrow indicates addition of 22 mg. quinol.

reaction rate made it necessary to lengthen the reaction time between successive additions of hydrogen peroxide so as to ensure full decomposition of each addition before making the next one.

DISCUSSION

The results reported above show that the addition of hydrogen peroxide to a wheat-germ suspension containing catalase, peroxidase and a substrate of the latter caused preferential oxidation of the peroxidase substrate. Catalase activity was largely suppressed until oxidation was complete. In the other systems studied, the extent of this suppression was found to depend on the concentration of the enzymes as well as on the ratio of their activities. Altschul & Karon (1947), referring to Kasanski's (1912) finding that catalase activity can be selectively destroyed by incubating the enzyme solutions with pyrogallol, used this method to destroy the catalase activity of cotton seed. The observations reported in this paper bear out the conclusion that pyrogallol, as well as other peroxidase substrates, damages catalase activity. The damage, however, did not occur in presence of peroxidase and substrates in concentrations up to 0·02 M.

Methods for catalase determination are based mainly on the establishment of the velocity constant for hydrogen peroxide decomposition. In systems containing any peroxidase substrates, including thiol compounds, the suppression of catalase activity by peroxidase might vitiate these methods. A similar complication affecting catalase activity might arise in presence of ascorbic acid and a phenolic substrate. In such a system the quinone formed by peroxidase would catalyse the oxidation of ascorbic acid. Under these conditions ascorbic acid would play the part of an additional substrate of peroxidase.

The method described in this paper offers possibilities for the detection of naturally occurring or added substrates of peroxidase.

SUMMARY

1. Hydrogen peroxide added to a wheat-germ suspension containing catalase, peroxidase and a substrate of the latter was preferentially used by peroxidase in the oxidation of the substrate. Catalase activity was largely suppressed while the oxidation of the substrate proceeded.

2. In the other systems studied, the extent of this suppression was found to depend on the concentration of the enzymes as well as on the ratio of their activities.

3. The peroxidase substrates tested damaged catalase activity, but were much less injurious in presence of peroxidase.

Our thanks are due to Mr D. G. Holliss for his assistance in the experimental part of this work.

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Studies on Hexokinase

1. THE HEXOKINASE ACTIVITY OF RAT-BRAIN EXTRACTS

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This and the two following papers are part of an investigation which was originally undertaken to study the possibility of using the hexokinase reaction for the assay of hormonal changes in human blood. For this purpose it seemed inadvisable to use purified hexokinase preparations, since the interaction with hormones might conceivably depend on the association of the enzyme with other tissue constituents. Preliminary experiments had shown that aqueous extracts of rat brain possessed a hexokinase activity which was higher, more stable and more reproducible than similar extracts of rat muscle. Moreover, these extracts were practically free of carbohydrate and of codehydrogenases and their apyrase activity was low. Undesirable side reactions were therefore less likely to occur. For these reasons most experiments were performed with an aqueous extract of rat brain as a source of hexokinase. In this paper the properties of the enzyme system used, together with some new observations on brain hexokinase, will be described.

METHODS AND MATERIALS

Abbreviations

ATP = adenosinetriphosphate; ADP = adenosinediphosphate; $P_{7\ min.}$ = P of acid-hydrolysable phosphate groups of ATP and ADP; terminal P = terminal (myosin-labile) P of ATP; HMP = hexosemonophosphate; HDP = hexosediphosphate.

Preparation of brain extracts

A rat brain was homogenized for 3 min. in a small glass homogenizer of close fit (Potter & Elvehjem, 1936) with a total volume of 7 ml. glass-distilled water, added in portions, at 0°. After centrifuging for 5 min. in an angle centrifuge at 3500 rev./min. the clear supernatant was taken off with a rubber-teat pipette, care being taken not to disturb the precipitate. The residue was suspended in 7 ml. water and again centrifuged. The combined extracts (approx. 11 ml.) contained about 6 mg. protein/ml. In the initial stages of the investigation the protein content of the extract was determined with Folin & Ciocalteu's (1927) reagent and the volume adjusted to the desired protein concentration. The colour formation from brain extracts with the phenol reagent had previously been calibrated by simultaneous Kjeldahl estimations. Since the protein content of the brain extracts was found to vary little, this estimation was later omitted. Extracts prepared in this way were found to retain their hexokinase activity for 2–3 days at 0°.

Composition of test solution

In all experiments inorganic constituents were added to give the following concentrations (M): $NaHCO_3$, 0·02; $MgCl_2$, 0·0067; NaF, 0·0238. The concentrations of brain extract, phosphate acceptor and phosphate donor were varied to suit the requirements of the experiment, but, unless otherwise stated, the standard concentrations were: brain extract, 0·2 ml. (approx. 1·2 mg. protein)/3 ml.; glucose, 1·85 mM; ATP, 3·33 mM.

The usual volume of the test solution was 3 ml., but for some experiments it was increased to 6 or 10 ml.

Experiments were carried out at 30° in an atmosphere of nitrogen containing 5 % (by vol.) CO_2.

Measurement of reaction rates

Manometric experiments. These were carried out in Warburg manometers. The reaction was started by tipping in the phosphate acceptor (glucose or HMP) from the side bulb; any pressure changes due to the reaction of ATP with preformed phosphate acceptors in the brain extract were thus avoided. With the small amounts of protein used, retention was found to be too small to be measured with accuracy. It was therefore neglected. Likewise, the 'esterification correction' (Meyerhof & Kiessling, 1933) was negligible since the K_2' values of the disappearing phosphate esters did not significantly differ from those of the newly formed esters.

Chemical estimations. The measurement of reaction rates by chemical methods involved the analysis of an initial and subsequent samples. The reaction was started by adding the brain extract as last component. The initial sample was withdrawn immediately afterwards with a pipette and delivered into a solution of the appropriate deproteinizing agent.

Analytical procedures

Glucose. Glucose was estimated in 0·5–1·0 ml. samples by the method of Nelson (1944) after $Ba(OH)_2$-$ZnSO_4$ precipitation. All estimations were carried out in duplicate.

Phosphate fractions. Inorganic phosphate was determined by the method of Fiske & Subbarow (1925) or, in later experiments, by a modification of the method of Martin & Doty (1949; see Weil-Malherbe & Green, 1951). The acid-labile phosphate groups of ATP and ADP were determined after hydrolysis in N-HCl at 100° for 7 min. and corrected for the phosphate fraction hydrolysed between 7 and 30 min. under the same conditions (Lohmann, 1928). Owing to the high yield of hexosediphosphate in these experiments serious errors were incurred if this correction was omitted.

ATP was determined from the phosphate hydrolysed by purified (four times precipitated) myosin; this was carried out as previously described (Weil-Malherbe, 1950) and

ADP was calculated by the difference: ($P_{7\,min.}$) – 2(myosin-labile P).

For the simultaneous estimation of inorganic, acid-labile and myosin-labile P, 4 ml. of test solution were added to 16 ml. 10 % (w/v) trichloroacetic acid. The filtrate was measured and 1 ml. N-HCl was added. The solution was extracted four times with 10 ml. portions of peroxide-free redistilled ether to remove excess trichloroacetic acid. Ether was removed *in vacuo* from the warmed solution which was then neutralized and made up to 20 ml. Inorganic P was determined in a 4 ml. sample and acid-labile P in two 1 ml. samples hydrolysed for 7 and 30 min. respectively. Two samples of 6 ml. were used for the estimation of myosin-labile P. Care was taken to avoid an excess of myosin, as this might have resulted in a partial hydrolysis of the second phosphate group of ATP. The correct amount of myosin was found in preliminary experiments with ATP solutions of known concentration.

Hexosediphosphate. HDP was determined by the estimation of alkali-labile phosphate after incubation with aldolase in presence of KCN (Meyerhof, Ohlmeyer & Möhle, 1938; Meyerhof & Junowicz-Kocholaty, 1942). Crystalline aldolase was prepared from rabbit muscle by the method of Taylor, Green & Cori (1948). The crystalline enzyme was dried *in vacuo* and 3 mg. of the powder, which contained some $(NH_4)_2SO_4$, were dissolved and added to a neutralized trichloroacetic acid filtrate. A calibration curve was determined with a solution of pure HDP.

The method estimates the sum of HDP and triosephosphates. All analytical results referring to 'HDP' represent in reality the sum of these compounds.

Ammonia. NH_3 was distilled in the apparatus of Parnas & Heller (1924) and determined by nesslerization.

Chemical preparations

ATP was prepared from rabbit muscle by the method of Dounce, Rothstein, Beyer, Meier & Freer (1948). A number of consecutive specimens showed a satisfactory ratio of total organic to acid-labile P, though the figures were higher than those calculated for $Ba_2(C_{10}H_{12}O_{13}N_5P_3).4H_2O$ (P, 10·92; $P_{7\,min.}$, 7·3 %), possibly due to the presence of some acid Ba salt. A specimen analysis was as follows: total organic P, 12·60; $P_{7\,min.}$, 8·36 %. The myosin-labile P usually amounted to 29–33 % of the total organic P.

ADP. After several unsatisfactory attempts with myosin a pure specimen of ADP was prepared with purified yeast hexokinase by incubating 30 mg. of enzyme-protein with 750 mg. glucose and 11·4 ml. 0·1 M-ATP in bicarbonate-CO_2 buffer (pH 7·4) for 30 min. at 30°. The volume of the solution, which also contained Mg^{++} in the usual concentration, was 150 ml. The ADP formed was purified through its mercuric and Ba salts in the usual way. Yield: 390 mg. Ba salt (total organic P, 8·7 %; $P_{7\,min.}$, 4·49 %).

The yeast hexokinase was obtained by the procedure of Berger, Slein, Colowick & Cori (1946) and corresponded to their fraction 3*a*. The bulk of the preparation which was not used for the preparation of ADP was dried after precipitation with $(NH_4)_2SO_4$ (0·7 saturation). The enzyme preparation obtained on solution and dialysis was highly active.

Hexosemonophosphates. A specimen of the neutral Ba salt of Robison ester mixture was given by Dr M. G. Macfarlane. Organic P: 8·08 % (on air-dried material).

A specimen of greater purity was isolated after the experiment in which ADP was prepared by adding an equal volume of ethanol to the supernatant from the first Ba precipitation. The air-dried Ba salt (340 mg.) contained 7·08 % organic P. Estimation of fructose (Roe, 1934) and aldose titration (Macleod & Robison, 1929) revealed a content of 45 % ketose and 55 % aldose.

Fructose-6-phosphate was prepared from HDP as the Ba salt according to Neuberg, Lustig & Rothenberg (1943). Organic P: 6·43 % (on air-dried material).

Glucose-6-phosphate was synthesized by the method of Lardy & Fischer (1946). The Ca salt, dried *in vacuo* at 78°, contained 10·69 % organic P.

Hexosediphosphate. The acid Ba salt was prepared from a commercial sample (Schwarz Laboratories, New York) according to Neuberg *et al.* (1943). It contained 12·80 % organic P.

All Ba salts were converted to the K salts for use. Ca glucose-6-phosphate was converted to the Na salt by passage through a column of Zeo-Karb 215 (Permutit Company Ltd., London), pretreated with Na^+.

Adenylic acid was prepared by alkaline hydrolysis of ATP (Lohmann, 1932). It contained 9·3 % organic P.

Cozymase, of purity 50–60 %, was prepared by the method of LePage (1947).

Insulin hydrochloride of low Zn content was obtained from British Drug Houses Ltd. and had: Zn, 0·035 %; activity, 20–21 units/mg.

Experimental error

A measure of the experimental error was obtained by the statistical analysis of fifty pairs of duplicate experiments in which hexokinase activity was determined by the rate of glucose disappearance. A standard deviation of 6·55 % was found. Deviations $\geqslant \pm 17$ % from the control experiment may therefore be regarded as significant ($P \leqslant 0{\cdot}01$).

RESULTS

The effect of substrate concentration

Glucose. The affinity between brain hexokinase and glucose is so great that the initial reaction rate (observed manometrically) is independent of the concentration of glucose above values of 0·1 mM. To obtain reliable results with smaller

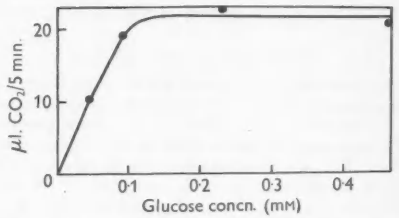


Fig. 1. Effect of glucose concentration on the initial rate of the hexokinase reaction. Manometric experiment: 0·1 ml. brain extract in 6 ml. Other components in standard concentration.

concentrations it was necessary to double the standard volume of solution per vessel and to reduce the standard concentration of brain extract to one-fourth. In this way the Michaelis constant, K_m, was estimated to be approximately 5×10^{-5} M (Fig. 1). This value is not very different from that of 1×10^{-4} M reported by Harpur & Quastel (1949).

Hexosemonophosphate. The Michaelis constant for the phosphorylation of hexosemonophosphate is appreciably greater than that for glucose. Values of K_m, obtained in several experiments, lay in the range $4–10 \times 10^{-4}$ M (Fig. 2) and are thus of an order similar to those obtained for fructose and D-glucosamine (Harpur & Quastel, 1949).

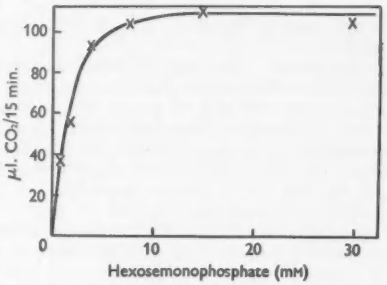


Fig. 2. Effect of hexosemonophosphate concentration on the initial activity of phosphohexokinase. ATP added: 20 μmol./3 ml. Other components in standard concentration.

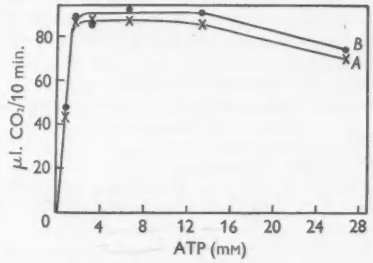


Fig. 3. Effect of ATP concentration on the initial rate of hexokinase and phosphohexokinase reactions. HMP added: 11·1 μmol./3 ml. Other components in standard concentration. Curve *A*, with glucose as substrate. Curve *B*, with HMP as substrate.

ATP. The maximum initial velocity of phosphorylation was reached with a concentration of 1·5–2 mM-ATP, with either glucose or hexosemonophosphate as phosphate acceptor (Fig. 3). This, however, was true only for the first 5–10 min. and, as the reaction progressed, the rate was maintained better if ATP was present in excess. Under the standard conditions of enzyme and glucose concentration, a concentration of 3·33 mM-ATP maintained the reaction at about its maximum rate for at least 20 min.

Reaction products

Manometric experiments. The manometric measurement of hexokinase activity in brain extracts is complicated by some side reactions, but the necessary corrections are small. The activity of apyrase was identical in the presence and in the absence of phosphate acceptors, as shown by the increase of inorganic phosphate (Table 1). A correction for the pressure changes due to apyrase activity can therefore be based on a control experiment without phosphate acceptors.

After the completion of the phosphorylation reaction a small negative pressure was usually observed. Similar pressure changes were observed when ADP or adenylic acid was added to brain extract. It was therefore attributed to the activity of adenylic deaminase.

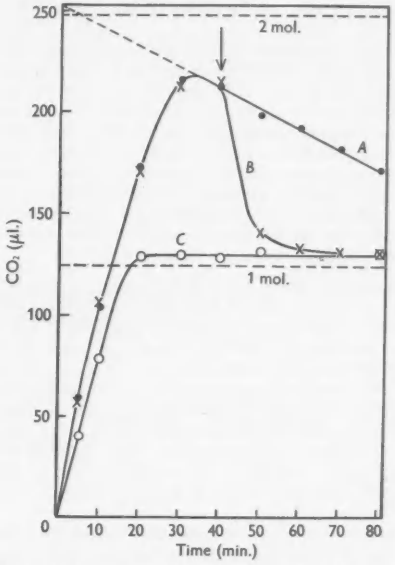


Fig. 4. Acid formation during hexokinase reaction. Brain extract, 0·4 ml.; ATP, 20 μmol.; glucose, 5·55 μmol. in 3 ml. Curve *A*, CO_2 evolution in presence of brain extract only. Curve *B*, addition of 1 mg. adenylic deaminase (fraction 3*P*, Weil-Malherbe & Bone, 1951*b*) after 40 min. Curve *C*, addition of 1 mg. adenylic deaminase at beginning of experiment.

The final yield of acid formation could be corrected for the activity of adenylic deaminase either by extrapolating the descending part of the curve back to zero time (see Figs. 4

Table 1. *End products of hexokinase and phosphohexokinase reactions*

(Brain extract 0·3 ml.; ATP, 20 μmol. in 3 ml. Duration of experiment, 50 min.)

Substrate added (μmol.)	Reaction products (μmol.)				
	CO_2	Inorganic P	NH_3	HDP	CO_2* + NH_3*
No addition	0·9	1·3	0·7	0	—
Glucose (5·55)	10·0	1·1	2·6	5·6	11·0
HMP (11·1)	9·8	1·2	2·7	9·3	10·9

* Corrected for control value without substrate.

and 7) or by the estimation of NH_3 formation, corrected for the NH_3 formation in a control experiment without phosphate acceptors (Table 1). With either method the result usually corresponded closely to the theoretical yield of 2 mol. acid in the case of glucose and of 1 mol. acid in the case of hexosemonophosphate.

Addition of a muscle fraction with high adenylic deaminase activity (fraction 3*P*, Weil-Malherbe & Bone, 1951*b*) after completion of the phosphorylation reaction resulted in an immediate rapid gas absorption. When the reaction was terminated the final acid formation amounted to 1 mol./mol. glucose (Fig. 4, curve *B*) in agreement with the reaction:

$$\text{Glucose} + \text{ATP} \rightarrow \text{HDP} + NH_3 + \text{inosinic acid.}$$

The same end value was reached when the muscle fraction was present from the beginning of the reaction (Fig. 4, curve *C*).

The CO_2 evolution was usually linear until the reaction was almost completed (Fig. 4, curve *A*), but an initial lag period, lasting for 10–20 min., was repeatedly observed, so that the resulting curve was slightly sigmoid in shape (Fig. 7, curve *A*).

If the reaction was stopped after 30–50 % of the glucose had disappeared, the ratio of acid formation to glucose disappearance was often significantly smaller than 2, probably indicating an intermediate accumulation of hexosemonophosphate. This decrease of the CO_2/glucose ratio was the more marked the greater the dilution of the brain extract (Table 2).

to a similar end value (Table 3) showing that isomerase activity was not a limiting factor in the brain extracts.

Formation of hexosediphosphate. Under the conditions of our experiments added hexosediphosphate was quantitatively recovered after incubation with brain extract. The rapid destruction of coenzyme I by the powerful nucleotidase of brain (McIlwain & Rodnight, 1949) effectively blocked glycolysis at the triosephosphate stage. The use of iodoacetate, advocated by Wiebelhaus & Lardy (1949), is therefore unnecessary.

The measurement of hexosediphosphate formation after the completion of the phosphorylation reaction indicated a quantitative conversion of both glucose and hexosemonophosphate to hexosediphosphate (Table 1), thus confirming the manometric experiments. On the other hand, lower yields were sometimes found when the reaction was stopped before it had gone to completion, especially when dilute brain extracts were employed (Table 2).

Phosphate utilization. The increase of inorganic phosphate in brain extracts was practically nil in the absence of ATP, even if hexosemono- or hexosediphosphate were added. In the presence of ATP, therefore, formation of inorganic phosphate may be attributed to apyrase activity. (The term apyrase is used to indicate the enzymic breakdown of ATP to adenylic acid and inorganic phosphate, even though it is possible that 'apyrase' activity is due to the combined action of ATPase and myokinase.) Accordingly the utilization of phosphate in the hexokinase reaction is obtained

Table 2. *Effect of enzyme concentration on ratios of carbon dioxide evolved and hexosediphosphate formed to glucose disappearing*

(ATP concn. 6·67 mM.)

Brain extract protein (mg./3 ml.)	μmol. Glucose added	Δ Glucose	Δ CO_2	Δ Inorganic P	Δ HDP	$-\Delta CO_2/\Delta$ glucose	$-\Delta$ HDP/Δ glucose
			Exp. 1. Duration 20 min.				
0·4	2·77	−0·83	1·0	0·12	0	1·20	—
1·2	5·55	−2·2	2·9	0·35	0·8	1·32	0·36
3·6	11·1	−6·4	10·8	0·50	4·2	1·69	0·66
			Exp. 2. Changes in first 20 min.				
1·2	5·55	−1·53	1·62	0·77	0·63	1·06	0·41
3·6	11·1	−4·88	8·15	0·97	4·9	1·67	1·0
			After completion of reaction (80 min.).				
1·2	5·55	−5·55	9·0	1·68	4·8	1·62	0·87
3·6	11·1	−11·1	18·5	3·80	11·0	1·67	0·99

Table 3. *Acid formation during phosphorylation of hexosemonophosphate*

(Brain extract, 0·3 ml.; ATP, 20 μmol. in 3 ml.)

	μmol. added	CO_2 formed (μmol.) In 10 min.	After completion
Hexosemonophosphate*	10·8	4·5	8·0
Glucose-6-phosphate	10·9	4·5	8·7
Fructose-6-phosphate	10·6	4·7	8·5

* Yeast-hexokinase preparation.

Equivalent amounts of hexosemonophosphate (yeast hexokinase preparation), fructose-6-phosphate and glucose-6-phosphate caused acid production at a similar rate and up

from (decrease of $P_{7\ min.}$ fraction) − (increase of inorganic P) and the utilization of ATP from (decrease of terminal P fraction) − 0·5 (increase of inorganic P). It must be emphasized again that it is essential to correct the phosphate hydrolysed after 7 min. by the increment of phosphate hydrolysis occurring between 7 and 30 min. as described.

The ratio of P utilized to glucose disappearance was close to the theoretical value of 2 in most experiments (Tables 4 and 5). It was of interest to know what proportion of the phosphate utilized originated from the terminal and from the intermediate phosphate group of ATP respectively. The calculation of the ADP formed during the reaction showed that in some experiments practically no ADP accumulated, but in others significant amounts were formed (Table 4). The brain extracts thus contain myokinase, whose activity is, however, not always sufficient to keep pace with the forma-

Table 4. *The phosphate balance of the hexokinase reaction in brain extracts*

(Brain extract, 0·9 ml.; glucose, 16·67 μmol.; ATP, 40 μmol. in 10 ml. Duration of experiment, 20 min.)

Exp. no.	Δ Glucose (μmol.)	Δ Inorganic P (μmol.)	Δ $P_{7\,min.}$ (μmol.)	P utilized* (μmol.)	Δ ATP (μmol.)	ATP utilized* (μmol.)	Δ ADP (μmol.)	P utilized/ Δ glucose
1	−7·2	1·08	−15·6	14·5	− 8·5	8·0	1·5	2·01
2	−9·9	3·2	−21·8	18·6	−11·0	9·4	0·2	1·88
3	−8·4	0·7	−18·3	17·6	−11·8	11·4	5·3	2·09

* Calculated as described in text.

Table 5. *Activity of ATP and ADP as phosphate donors in the hexokinase reaction*

(Brain extract, 0·2 ml.; glucose, 5·55 μmol.; ATP, 10 μmol. or ADP, 20 μmol. in 3 ml. Duration of experiment, 20 min.)

Exp. no.	Phosphate donor	Δ Glucose (μmol.)	Δ Inorganic P (μmol.)	Δ $P_{7\,min.}$ (μmol.)	P utilized (μmol.)	NH_3* (μmol.)
1	ATP	−2·7	1·6	−7·0	5·4	1·06
	ADP	−2·0	1·0	−4·6	3·6	2·12
2	ATP	−3·3	—	—	—	—
	ADP	−2·0	—	—	—	—
3	ATP	−3·8	—	—	—	—
	ADP	−2·7	—	—	—	—

* In 60 min.

tion of ADP by hexokinase. If ATP is replaced by ADP as phosphate donor the rate of the reaction is reduced by about 30 % (Table 5). This may be due to an inhibitory effect of ADP or to the fact that, in absence of ATP, the reaction rate is limited by the activity of myokinase. It is, however, unlikely that myokinase activity was limiting in presence of ATP, as the latter was always added in considerable excess. Moreover, at the end of the experiment, about 70 % of the amount initially added could be recovered (Table 4).

Ammonia formation. In absence of phosphate acceptors about 1 mol. of NH_3 was formed for 2 mol. of inorganic P. NH_3 formation in presence of phosphate acceptors was only slightly higher (Tables 1 and 5), indicating a fairly weak activity of adenylic deaminase. Similar amounts of NH_3 were formed from adenylic acid and from ADP (Table 6); myokinase activity was therefore not a limiting factor for ammonia formation.

Table 6. *Ammonia formation from ADP and adenylic acid in brain extract*

(Brain extract, 0·2 ml.; ADP or adenylic acid, 10 μmol. in 3 ml. Duration of experiment, 80 min.)

	Δ NH_3 (μmol.)
ADP	2·7
Adenylic acid	2·9

The activities of apyrase, myokinase and adenylic deaminase were somewhat variable in different extracts. Moreover, they seemed to vary in parallel. According to Meyerhof & Geliazkowa (1947) brain apyrase is largely bound to insoluble tissue particles. If the same is true for myokinase and adenylic deaminase the observed variations may be explained by the varying efficiency of comminution, centrifugation and the separation of the supernatant. Though the procedure was standardized as far as possible, a complete control of these factors was apparently not achieved.

Inhibition by reaction products

According to Colowick & Kalckar (1943) yeast hexokinase is not inhibited by either ADP, adenylic acid or hexosemonophosphate, while myokinase is inhibited by adenylic acid. We have fully confirmed these observations. But in contrast to yeast hexokinase the hexokinase of brain extracts is strongly inhibited by hexosemonophosphate in low concentrations. In spite of the presence of isomerase it was found in experiments of short duration that glucose-6-phosphate is more inhibitory than fructose-6-phosphate and that the inhibitory effect of the natural equilibrium mixture is about half-way between those of the pure components (Table 7),

Table 7. *Effects of hexosemonophosphates and adenylic acid on the hexokinase activity of brain extracts*

(Brain extract, glucose and ATP in standard concn.; duration of experiment, 20 min.)

Addition	Concn. (mM)	Decrease in glucose (mg.)	Inhibition (%)
None	—	0·473	—
Hexosemonophosphate*	0·55	0·398	16
	1·83	0·263	44·5
Fructose-6-phosphate	0·55	0·430	9
	1·83	0·360	24
Glucose-6-phosphate	0·55	0·347	26·5
	1·83	0·140	72
None	—	0·180	—
Adenylic acid	3·33	0·203	0

* Yeast-hexokinase preparation.

suggesting that the effect is due solely to glucose-6-phosphate. As regards the different response of brain and yeast hexokinase, it may be recalled that adrenochrome inhibits the former but not the latter (Meyerhof & Randall, 1948).

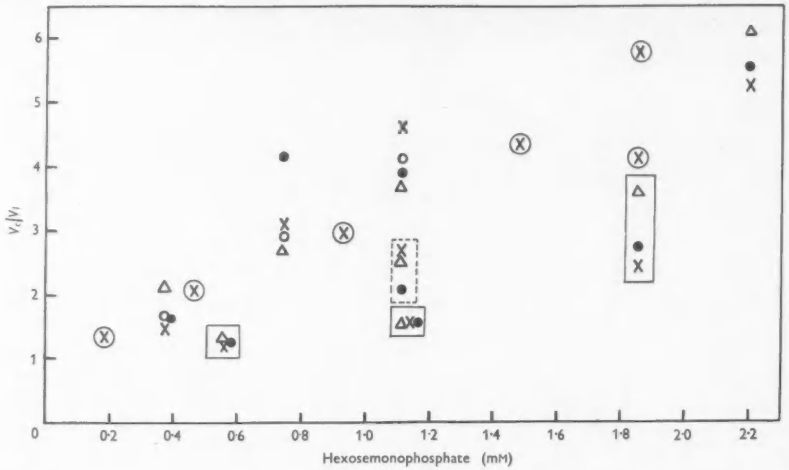


Fig. 5. Effect of glucose concentration on the inhibition of brain-hexokinase activity by increasing concentrations of hexosemonophosphates. Brain extract, 0·2 ml.; ATP, 10 μmol. in 3 ml. The figure includes the results of several experiments. Exp. 1 (unenclosed symbols): hexosemonophosphate. Exp. 2 (symbols in circles): hexosemonophosphate. Exps. 3–6 (symbols in rectangles): fructose-6-phosphate. V_e=reaction rate of control, V_I=reaction rate in presence of inhibitor. ○, 0·370 mM-glucose; △, 0·925 mM-glucose; ×, 1·85 mM-glucose; ●, 3·70 mM-glucose.

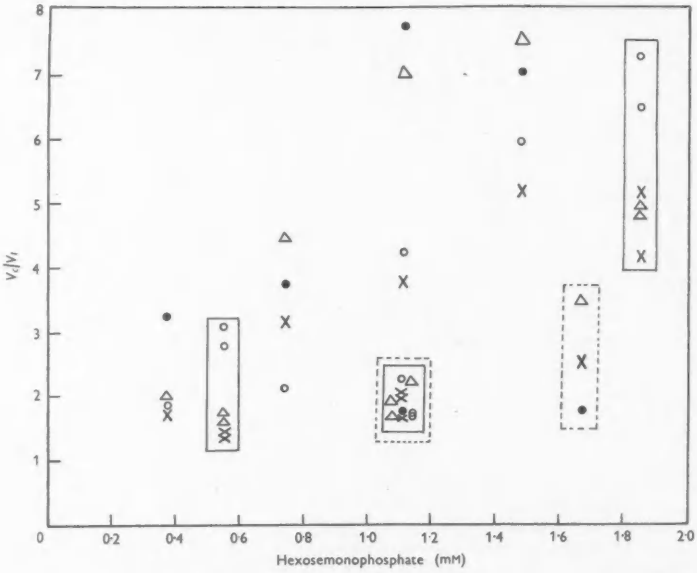


Fig. 6. Effect of ATP concentration on the inhibition of brain-hexokinase activity by increasing concentrations of hexosemonophosphates. Brain extract, 0·2 ml.; glucose, 5·55 μmol. in 3 ml. Exp. 1 (unenclosed symbols): hexosemonophosphate. Exp. 2 (symbols in full-line rectangle): fructose-6-phosphate. Exp. 3 (symbols in dotted-line rectangle): fructose-6-phosphate. V_e, V_I as in Fig. 5. ○, 1·67 mM-ATP; △, 3·33 mM-ATP; ×, 6·67 mM-ATP; ●, 10·0 mM-ATP.

A graphical analysis according to the method of Ebersole, Guttentag & Wilson (1944) showed that the inhibition is non-competitive with respect to either glucose or ATP (Figs. 5 and 6). With concentrations of ATP < 2 mM an increase of inhibition was sometimes found compared with higher concentrations of ATP, but above this level the inhibitory effect did not consistently vary with ATP concentration. At the lower levels of ATP concentration the saturation of hexokinase and phosphohexokinase was presumably incomplete resulting in a slower rate of hexosemonophosphate disappearance.

There was no inhibition of hexokinase by adenylic acid (Table 7), or of hexokinase or phosphohexokinase by hexosediphosphate. The absence of an inhibitory effect of adenylic acid strengthens the conclusion that, under the conditions employed, the reaction rate is not limited by myokinase activity.

Summation experiments

When the phosphorylation of glucose, hexosemonophosphate and of both together was measured by the manometric method, the initial reaction rates were about equal (Fig. 7).

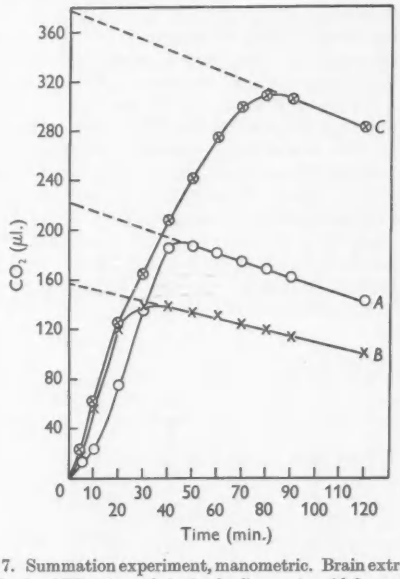


Fig. 7. Summation experiment, manometric. Brain extract, 0·3 ml.; ATP, 20 μmol. in 3 ml. Curve *A*, acid formation during phosphorylation of 5·5 μmol. glucose. Curve *B*, acid formation during phosphorylation of 7·2 μmol. glucose-6-phosphate. Curve *C*, acid formation in presence of both substrates.

There was thus no summation. Two explanations are possible: either the two phosphorylation reactions are limited by the activity of a common factor or the phosphorylation of glucose is virtually suspended until most of the inhibitory glucose-6-phosphate has been removed. The only factor known to be common to both hexokinase and phosphohexokinase is ATP, which was present in sufficient excess to saturate both systems. That the second explanation is probably correct is indicated by the course of glucose disappearance as determined chemically in a parallel experiment; it shows that the reaction was strongly inhibited so long as hexosemonophosphate was present (Fig. 8).

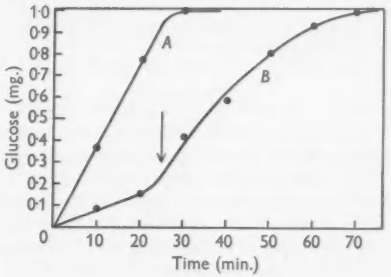


Fig. 8. Summation experiment; chemical estimation of glucose disappearance. Conditions as in experiment of Fig. 7. Curve *A*, glucose disappearance in absence of glucose-6-phosphate. Curve *B*, glucose disappearance in presence of 7·2 μmol. glucose-6-phosphate. The arrow indicates the completion of glucose-6-phosphate phosphorylation in the simultaneous manometric experiment.

The phosphorylation of hexosemonophosphate in preference to glucose may seem in disagreement with the fact that the affinity of glucose for the phosphorylating enzyme is higher than that of hexosemonophosphate. In reality, no such contradiction exists, as the inhibition by hexosemonophosphate was found to be non-competitive and therefore independent of the relative affinities of the two substrates. Furthermore, while the inhibitory compound, glucose-6-phosphate, reacts with hexokinase, the substrate of phosphorylation, fructose-6-phosphate, reacts with a different enzyme, phosphohexokinase.

Activity of rat-brain hexokinase in presence of various substances

In view of the activation of hexokinase by proteins from erythrocytes and muscle tissue (Weil-Malherbe & Bone, 1951 *a*, *b*) it was necessary to investigate the specificity of the effect. A number of substances were therefore tested for their effect on hexokinase activity. Those selected were known to have a protective effect on enzymes in general, either by their colloidal nature, by their combination with heavy metals or by their reducing properties. The first group included glycogen, gum arabic and several proteins, such as casein, horse serum (dialysed against distilled water and inactivated at 56° for 4 hr.), and ovalbumin, both before and after heat denaturation; the second group consisted of cyanide, pyrophosphate and the amino-acids histidine and glycine; and the third group of the thiol compounds cysteine and glutathione, and ascorbic acid. None of these substances caused any signficant activation.

In all experiments ATP was added in the form of the K salt. The concentration of K^+ was therefore 0·0132 M. Any further increase of the K concentration, or complete replacement of Na by K was without effect on hexokinase activity.

In addition, certain substances were tested, which were reported, or may be suspected, to have activating effects on hexokinase. But in our experiments no activation was observed with cozymase or reduced cozymase (Colowick & Price, 1945*a*), both in presence and absence of nicotinamide, with guanine (Colowick & Price, 1945*b*), with insulin, with acetylcholine or with various combinations of these compounds. In view of the negative results of these experiments, the actual figures are omitted.

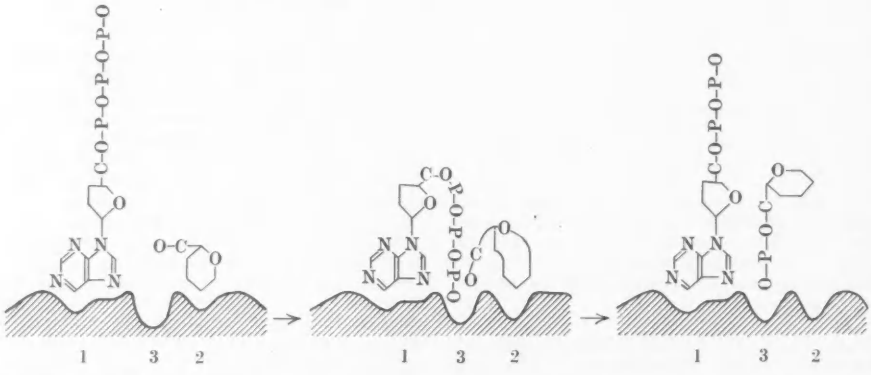
DISCUSSION

The brain extracts used in these experiments were shown to convert glucose quantitatively to hexosediphosphate. In addition to hexokinase and phosphohexokinase they contain small and somewhat variable amounts of 'apyrase', myokinase and adenylic deaminase resulting in the formation of adenylic and inosinic acids from ATP and ADP. The myokinase activity is usually sufficiently great to ensure at least a partial utilization of the intermediate phosphate group of ATP, but, except perhaps with ADP as phosphate donor, the reaction rate was not limited by the activity of myokinase owing to the presence of a sufficient excess of ATP.

There are indications that in the initial phases of the reaction, and especially with more dilute extracts, some hexosemonophosphate accumulates. But this accumulation is unlikely to go far as it would soon cause inhibition of hexokinase activity and a preferential phosphorylation of hexosemonophosphate.

These results differ in some respects from those obtained by Meyerhof and his colleagues in similar experiments. Meyerhof & Geliazkowa (1947) found apyrase activity to be greater in the absence than in the presence of phosphate acceptors, whereas we found it to be the same in both cases. The difference is probably due to the fact that we used an excess of ATP (10–20 μmol.) over glucose (5·5 μmol.), while in Meyerhof & Geliazkowa's experiments glucose (22 μmol.) was present in excess (initial concentration of ATP 3–4 μmol.). Meyerhof & Geliazkowa also claim, on indirect evidence, that ADP was practically stable in their brain extracts owing to the absence of myokinase. Yet in later experiments (Meyerhof & Wilson, 1948) it was shown that ADP was utilized as phosphate donor with the same efficiency as ATP. Finally, Meyerhof & Randall (1948) assume that 'nothing but HMP is formed at first with an excess of glucose'. This conclusion was based on the observation of a ratio of P utilization to glucose disappearance of 1, but the figures for P utilization were not corrected for the simultaneous hydrolysis of hexosediphosphate by subtracting the increment of phosphate hydrolysis occurring between 7 and 30 min. from the 7 min. hydrolysis. Relying on this statement we also at first omitted the correction and sometimes found ratios close to 1. When the correction was applied, however, substantially higher ratios were obtained and we are therefore inclined to regard the earlier observations as fortuitous.

Since the inhibition of hexokinase by hexosemonophosphate is, on the whole, independent of the concentration of either glucose or ATP, it is of a non-competitive nature. According to current interpretations of the mechanism of enzyme action this means that hexosemonophosphate combines with the enzyme at a site different from those which react with either glucose or ATP, but which is nevertheless essential for the reaction. If we designate this site by the number 3, and the sites reacting with ATP and glucose by 1 and 2, respectively, the mechanism of action of brain hexokinase may perhaps be envisaged as taking place in the following stages:



ATP is assumed to react with site 1 in such a way that its terminal phosphate group is free; similarly, glucose is assumed to react with site 2 in such a way that the C-6 group is free. The free groups of both

molecules meet at site 3 and there react to form glucose-6-phosphate. If site 3 is already occupied by glucose-6-phosphate, reaction cannot occur. ATP at site 1 is converted to ADP as a result of the reaction and on the basis of the scheme one might expect a competitive inhibition of brain hexokinase by ADP, reversible by increasing concentrations of ATP. This point can only be tested with preparations of animal hexokinase free of myokinase. It is hoped to investigate this problem in the future.

SUMMARY

1. The reactions occurring in aqueous rat-brain extracts during the phosphorylation of glucose by adenosinetriphosphate (ATP) and the properties of rat-brain hexokinase have been investigated.

2. Estimation of the Michaelis constant yielded approximate values of 5×10^{-5}M for hexokinase and 7×10^{-4}M for phosphohexokinase. The affinity for ATP was the same for both enzymes. The Michaelis constant in this case was about 8×10^{-4}M.

3. A study of the reaction products showed that glucose was quantitatively converted to hexosediphosphate and that glycolysis did not, under the conditions used, proceed beyond the triosephosphate stage. In the initial stages of the reaction some hexosemonophosphate may accumulate, especially when the enzyme is diluted.

4. The brain extracts used contained somewhat variable amounts of apyrase, myokinase and adenylic deaminase. The activity of myokinase was sufficient to prevent the accumulation of any but small amounts of adenosinediphosphate (ADP). When ADP was used as phosphate donor the reaction rate was about 30% slower than in the presence of ATP. But since ATP was always present in excess, even at the end of experiments, the activity of myokinase was never a limiting factor in the reaction. The ammonia formation from ATP, in absence of phosphate acceptors, was limited by the activity of apyrase rather than adenylic deaminase, while the ammonia formation from ADP was limited by the activity of adenylic deaminase rather than myokinase.

5. Brain hexokinase was strongly inhibited by hexosemonophosphates. Glucose-6-phosphate was found to be more inhibitory than fructose-6-phosphate, while Robison ester has an effect intermediate between the two components. The results suggest that the inhibitory compound is glucose-6-phosphate. The inhibition by hexosemonophosphates was non-competitive with respect either to glucose or ATP.

6. No summation of initial reaction rates of hexokinase and phosphohexokinase was observed when glucose and hexosemonophosphate were simultaneously added to brain extract, because hexokinase activity was inhibited in presence of hexosemonophosphate.

7. The addition of various substances known to have protective or activating effects on enzymes in general and on hexokinase in particular, alone or in combination, had no effect on the hexokinase activity of rat-brain extracts.

Our thanks are due to Dr R. Ström-Olsen, Physician Superintendent, for his interest and encouragement and to Dr Marjorie G. Macfarlane and Dr H. Waelsch for generous gifts of hexosephosphates.

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Studies on Hexokinase

2. AN ACTIVATOR OF HEXOKINASE IN ERYTHROCYTES

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The observation that cytolysates of washed human erythrocytes strongly enhanced the hexokinase activity of rat-brain extracts was made in the course of experiments undertaken to investigate the effects of constituents of human blood on hexokinase. The erythrocyte effect has briefly been mentioned in preliminary reports (Weil-Malherbe, 1948, 1949, 1950).

METHODS

Preparation of erythrocyte lysates. Blood containing 0·3 % NaF as anticoagulant was centrifuged, and the packed corpuscles were washed three times with 0·9 % (v/v) aqueous NaCl. Water was added up to the original blood volume and cytolysis was completed by freezing. The solution will henceforth be referred to as haemolysate. Unless otherwise stated, haemolysates were prepared from human blood.

Measurement of hexokinase activity. The experimental arrangements, analytical methods and preparations used were described in the preceding paper (Weil-Malherbe & Bone, 1951*a*). The standard test solution contained brain extract, glucose, adenosinetriphosphate (ATP) and mineral constituents in the concentrations stated, and only deviations from the standard conditions are expressly mentioned in the following sections. Haemolysate, when present, was added in amounts of 1 ml./3 ml. of final volume.

Hexokinase activity was usually measured by the rate of disappearance of glucose, and activation effects have been expressed in relation to the activity in the control experiment, designated by 100.

In manometric experiments containing haemolysate retention of CO_2 was determined and an appropriate correction applied.

RESULTS

Distribution and specificity of the activator

Frequency distribution. Fig. 1 shows the frequency distribution of the effects obtained with over 300 different human blood samples. The peak of the curve corresponds to a doubling of the activity of the control, while the highest effect observed was a sixfold increase in activity. On the other hand, the effect was frequently absent and some samples even caused inhibition. It has been found in experiments, which will be described elsewhere, that fresh human plasma may have a marked effect on hexokinase activity, depending, amongst other factors, on a concurrent assimilation of carbohydrate. It is assumed that these effects are of hormonal origin. Erythrocyte lysates prepared from blood samples from the same subject, withdrawn in the fasting

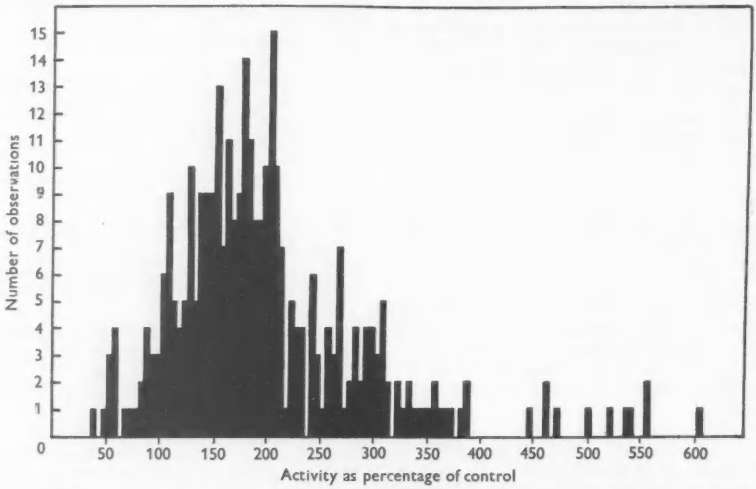

Fig. 1. Frequency distribution of the activation effect of human haemolysates on brain hexokinase.

state and at various intervals after administration of glucose, often show changes in their effect on hexokinase, which are parallel to the effects of plasma. But while the variations of hexokinase activity due to the addition of plasma are centred around the level of the control experiment, those due to haemolysates are centred around a higher level of activity. It seems as if the variability of the effects observed with fresh haemolysates were due, at least in part, to these supposedly hormonal effects which are superimposed on the effect of an activator of hexokinase present in red cells. This paper is concerned with the effects and properties of the activator only.

Effect of concentration. On increasing the concentration of the haemolysate the activation effect at first increases steeply, but finally appears to approach saturation (Fig. 2).

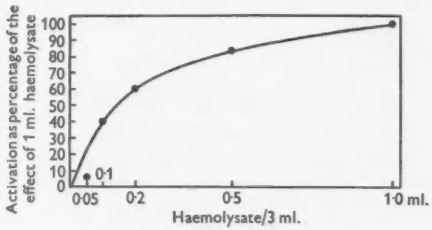

Fig. 2. Effect of haemolysate concentration on the activity of rat-brain hexokinase.

Species differences. Three species other than man, i.e. horse, rabbit and rat, were investigated. Two specimens of horse blood had high activity, but the effect seemed to be less marked and less constant with haemolysates from rat and rabbit (Table 1).

Table 1. *Effect of haemolysates from different animals on the activity of rat-brain hexokinase*

	Hexokinase activity (activity of control = 100)
Horse, 1	246
Horse, 2	350
Rabbit, 1	118
Rabbit, 2	162
Rat, 1	180
Rat, 2	115

Activation of muscle and yeast hexokinases. The activation effect is not confined to brain hexokinase, but may also be demonstrated with muscle extract and with a purified yeast hexokinase (Table 2). The response of these enzyme preparations is, however, smaller than that observed with brain extract.

Activation of fructose phosphorylation. Addition of haemolysate to rat-brain extract activates not only the phosphorylation of glucose, but also that of fructose (Table 3). On the other hand, the activity of phosphohexokinase is probably not changed, as will be shown later.

Properties of the activator

Effect of dialysis. Thorough dialysis of haemolysates was carried out in cellophan sacs against frequently changed distilled water at 0° until the volume remained constant. Compared with an undialysed portion of the same sample, similarly diluted, the activity was practically unchanged. A similar result was obtained after exhaustive electrodialysis (Table 4). Conversely, ultrafiltrates prepared with the aid of collodion sacs, and concentrated diffusates were inactive. Untreated ultrafiltrates and diffusates were even inhibitory, but they could be freed from inhibitory material (Fe ions?)

Table 2. *Effect of haemolysate on the activity of muscle and yeast hexokinases*

(Muscle extract: cooled rat muscle ground in Latapie mill, stirred with 1·5 vol. 0·033 M-$NaHCO_3$ for 15 min. at 0°. Centrifuged. Supernatant diluted with equal volume of glass-distilled water. Yeast hexokinase solution: 0·1 mg. protein/ml.)

Enzyme	Amount taken (ml.)	Decrease in glucose (mg.) Without haemolysate	With haemolysate	Activity* (control = 100)
Brain	0·2	0·400	0·815	177
Muscle	0·8	0·265	0·440	136
Yeast	0·2	0·480	0·714	132
Haemolysate	1·0	0·060	—	—

* Corrected for the hexokinase activity of the haemolysate.

Table 3. *Effect of haemolysate on the phosphorylation of fructose by rat-brain extract*

(Fructose: 5·55 μmol./3 ml. Other constituents in standard concentration.)

	Decrease in fructose (mg.)		Activation* (activity of control = 100)	
	Exp. 1†	Exp. 2‡	Exp. 1	Exp. 2
Brain	0·270	0·200	—	—
Haemolysate	0·040	0·010	—	—
Brain + haemolysate	0·400	0·358	129	171

* Corrected for activity of haemolysate.
† Fructose estimation by method of Roe (1934).
‡ Fructose estimation by method of Nelson (1944).

by passage through a column of Na^+-saturated resin (Zeo-Karb 215). The small loss of activity sometimes observed after prolonged dialysis was unaffected by returning the concentrated diffusate (Table 4).

Table 4. *Activating effect of dialysed and undialysed haemolysates*

(Undialysed sample diluted to match the haemoglobin concentration of the dialysed sample.)

Haemolysate specimen no.	Hexokinase activity of brain extract (activity of control = 100)	
	With undialysed haemolysate	With dialysed haemolysate
1	332	266
2	144	146
3	350	333*
4	163	162*
5	233	212
		(Same + concentrated dialysate) 209

* Electrodialysed.

Activity of stroma and haemoglobin. The activation effect was not associated either with human corpuscle stroma or with crystalline oxyhaemoglobin of man, horse or rat (Table 5). Some samples of crystalline haemoglobin produced activation, but since the effect was much smaller than that of the complete haemolysate it may be attributed to incomplete removal of the activating factor.

Corpuscle stroma was prepared by repeated washings of the centrifuged residue of a human haemolysate (500 ml.) with 0·033 M-sodium citrate buffer (pH 5·5) (Bernstein, Jones, Erickson, Williams, Avrin & Macy, 1937). The pinkish residue was finally homogenized in 0·05 M-$NaHCO_3$ (30 ml.).

the fraction precipitated between 0·3 and 0·5 saturation. These experiments are therefore not being reported in detail.

Some of the $(NH_4)_2SO_4$ fractions were spectroscopically free of haemoglobin, although they contained the activating factor. This confirms the non-identity of the activating factor and haemoglobin.

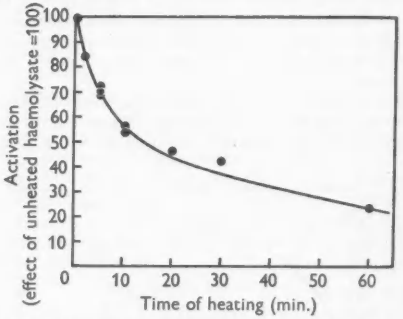

Fig. 3. Heat stability of the activating factor of haemolysates. Samples were heated in a water bath at 100°, cooled and homogenized.

Heat stability. The activating factor of the haemolysate is comparatively stable to heat. In Fig. 3 the time of heating at 100° is plotted against the activating effect expressed as a percentage of the effect caused by the unheated haemolysate. After 5 min. 80 %, and even after 60 min. 20 % of the original effect was still preserved. Though these results were repeatedly obtained, it occasionally happened, for unexplained reasons, that the activating effect was more markedly diminished or even completely lost after heating at 100° for 5 min.

Table 5. *Effect of crystalline haemoglobin and of erythrocyte stroma on hexokinase activity of rat-brain extracts*

	Hexokinase activity of brain extract (activity of control = 100)	
	Plus solution of crystalline haemoglobin	Plus complete haemolysate of same haemoglobin concentration
Human oxyhaemoglobin, 1	107	145
Human oxyhaemoglobin, 2	120	155
Horse oxyhaemoglobin, 1	152	246
Horse oxyhaemoglobin, 2 (recrystallized)	176	350
Rat oxyhaemoglobin	129	174*
Human washed stroma	94	145

* Supernatant, after removal of haemoglobin crystals.

Crystalline human oxyhaemoglobin was prepared according to Drabkin (1949) and horse oxyhaemoglobin according to Taylor & Hastings (1939). The crystals were dissolved in 0·05 M-$NaHCO_3$ at 37° and the solution diluted to match the concentration of the complete haemolysate.

Other fractionation experiments. Attempts to purify the activating factor by isoelectric precipitation or $(NH_4)_2SO_4$ fractionation were disappointing as the activity was spread out over a number of fractions with only a slight peak, which in the case of $(NH_4)_2SO_4$ fractionation appeared in

Even after heating, the activating factor was closely associated with protein. When a fresh haemolysate was heated and separated into supernatant and coagulum (which was well washed and homogenized in the original volume of distilled water) the activity of the two fractions was about the same and was equal to that of the whole of the heated haemolysate (Table 6). The supernatant used in this experiment was turbid and gave a precipitate with trichloroacetic acid. Clear supernatants containing only traces of protein were obtained when the haemolysate was dialysed,

Table 6. *Activating effect of fractions of heated haemolysate on hexokinase activity of rat-brain extract*

(Samples were heated in water bath at 80° for 10 min.)

	Activity in presence of fraction (activity of control = 100)
Haemolysate, unheated	287
Heated haemolysate, homogenized	224
Heated haemolysate, supernatant	218
Heated haemolysate, washed coagulum	228
Dialysed haemolysate, unheated	248
Dialysed haemolysate, heated, supernatant	113
Dialysed haemolysate, heated, washed coagulum	187
Diffusate, concentrated	105

Table 7. *Activating effect and hexokinase activity of haemolysates*

	Decrease in glucose (mg.)		
	Exp. 1	Exp. 2	Exp. 3
Brain extract	0·480	0·235	0·485
Haemolysate	0·070	0·040	0·100
Brain extract + haemolysate	0·892	0·625	0·835
Brain extract	0·650	—	—
Haemolysate, dialysed	0·010	—	—
Brain extract + dialysed haemolysate	1·000	—	—
Brain extract	0·320	0·560	—
Haemolysate	0·060	0·075	—
Haemolysate, heated (5 min. at 60°)	0	0	—
Brain extract + haemolysate	0·590	0·780	—
Brain extract + heated haemolysate	0·550	0·715	—

either before or after being heated. Such a supernatant had no activating effect (Table 6). Various other attempts to split off or extract the activating factor from denatured protein were unsuccessful: there was no activity in a concentrated diffusate from heated haemolysate (Table 6), in a trichloroacetic acid extract of red cells or in extracts prepared by heating haemolysates in NaCl solutions of varying strength (1–10 % w/v), followed by filtration and dialysis of the filtrate.

The mechanism of the activating effect

The facts described strongly suggest that the activator of haemolysates is either itself a protein or else a factor which cannot be separated from proteins even after their denaturation. Its heat stability is limited and is therefore not inconsistent with its protein nature. But before a new principle is assumed the question must be examined if the effects can be attributed to any of the known catalysts of glycolysis or associated reactions. The following experiments show that the effect of even the unheated haemolysate cannot be adequately explained by the activity of several such enzymes present.

Hexokinase activity of haemolysates. The hexokinase activity of haemolysates prepared in the manner described was low and could in no way account for the activation of brain hexokinase (Table 7). Moreover, haemolysates could be deprived of hexokinase activity by heating at 60° for 5 min. or simply by ageing. The decrease of activity after thorough dialysis (Table 7) is probably an effect of ageing. The activating properties of the haemolysate were not affected by these procedures.

It has been tacitly assumed so far that the hexokinase of brain extracts is activated by a factor present in the haemolysate. The reverse possibility, i.e. activation of erythrocyte hexokinase by a factor present in brain extract, was investigated by adding brain 'Kochsaft' and brain extract, heated at 60° for 5 min., to haemolysate. These additions, however, did not increase the hexokinase activity of haemolysates.

Phosphohexokinase activity of haemolysates. In contrast to their slight hexokinase activity haemolysates contain a very active phosphohexokinase which retains part of its activity after heating for 10 min. at 60°. The addition of haemolysate to brain extract results in an increase of phosphohexokinase activity which is, however, fully accounted for by the activities of the separate components (Table 8). The effect of the activating factor of haemolysates on brain phosphohexokinase can better be studied after heating the haemolysate for 5 min. at 80°, a treatment which completely inactivates phosphohexokinase. The experiment represented by Fig. 4 shows that, while the hexokinase activity of the brain extract was markedly activated by the heated haemolysate, there was no such effect on phosphohexokinase activity. This suggests that the activating factor specifically acts on hexokinase and that the effect cannot be attributed to a more rapid removal of hexosemonophosphates and to a relief of the inhibition caused by them (Weil-Malherbe & Bone, 1951*a*). In fact, a sample of haemolysate which had been heated to inactivate phosphohexokinase had no significant effect on the inhibition of hexokinase by hexosemonophosphate, although the uninhibited part of hexokinase was activated to about the same extent (Table 9). In any case, it has already been shown (Weil-Malherbe & Bone, 1951*a*) that the brain extracts used quantitatively convert glucose to hexosediphosphate and that there is no evidence that hexosemonophosphate accumulates to such an extent that inhibitory concentrations are reached. Even if it is assumed that inhibitory concentrations of hexosemono-

Table 8. *Phosphohexokinase activity of brain extract, of haemolysate and of both together*

(Manometric experiment. Hexosemonophosphate, 20 μmol.; ATP, 20 μmol./3 ml.)

	CO_2 evolution in 15 min. (μl.)
Brain extract	151
Haemolysate	188
Haemolysate, heated at 60° for 10 min.	144
Brain extract + haemolysate	268
Brain extract + heated haemolysate (60°, 10 min.)	260

phosphates do accumulate, the effect could only be manifested some time after the start of the reaction. The full activating effect of the haemolysate is, however, displayed immediately.

Triosephosphate dehydrogenase activity of haemolysates. Triosephosphate dehydrogenase activity leads to resynthesis of ATP. This might be an important factor if the concentration of ATP were limiting.

Untreated fresh haemolysates possess considerable triosephosphate dehydrogenase activity, as shown by the disappearance of added hexosediphosphate. The activity disappears after dialysis or in presence of iodoacetate (Table 10). The activating effect is, however, not influenced by either treatment (Tables 4 and 11), showing that it is unconnected with triosephosphate dehydrogenase activity.

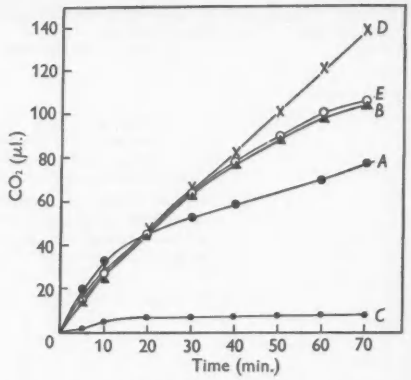

Fig. 4. Activating effect of heated haemolysate on brain hexokinase and phosphohexokinase. Brain extract, 0·1 ml.; ATP, 20 μmol.; glucose or hexosemonophosphate (HMP), 5·55 μmol./3 ml. Haemolysate heated at 80° for 5 min. Curve *A*, brain extract, glucose. Curve *B*, brain extract, HMP. Curve *C*, haemolysate, glucose; haemolysate, HMP. Curve *D*, brain extract + haemolysate, glucose. Curve *E*, brain extract + haemolysate, HMP.

Table 9. *Effect of heated haemolysate (5 min. at 80°) on the inhibition of brain hexokinase by hexosemonophosphate*

Fructose-6-phosphate concentration (mM)	Brain extract: Decrease in glucose (mg.)	Brain extract: Inhibition (%)	Brain extract + haemolysate: Decrease in glucose (mg.)	Brain extract + haemolysate: Inhibition (%)	Brain extract + haemolysate: Activating effect (control = 100)
0	0·298	—	0·545	—	183
0·55	0·185	38	0·282	48	—
1·11	0·092	69	0·208	62	—
1·85	0·044	85	0·180	67	—

Table 10. *Disappearance of hexosediphosphate in haemolysate*

(Hexosediphosphate, 10 μmol./3 ml. Haemolysate, mineral constituents and ATP in standard concentration. Duration of experiment, 30 min.)

	Δ HDP (μmol.)
Haemolysate	−2·33
Haemolysate + iodoacetate (1 mM)	+0·50
Haemolysate, dialysed	±0

Myokinase. It has been shown (Weil-Malherbe & Bone, 1951 *a*) that the brain extracts used in these experiments have considerable myokinase activity, since the second phosphate group of ATP as well as added adenosinediphosphate (ADP) are utilized in the reaction. The myokinase activity of the haemolysate, on the other hand, appeared to be low (Table 12). If the activation effect were due to the myokinase activity of the haemolysate one would expect the utilization of ADP to equal that of ATP. Yet, although the reaction is activated by the haemolysate, whether ADP or ATP is used as phosphate donor, the difference in the utilization of the two compounds persists (Table 13). Table 14 shows, furthermore, that a fourfold increase of ATP concentration neither

Table 11. *Effect of iodoacetate on rat-brain hexokinase in presence and in absence of haemolysate*

	Hexokinase activity (activity of control = 100) in presence of iodoacetate (mM): 0	0·33	1·0	3·3	10
Brain extract	100	93	82	77	62
Brain extract + haemolysate	269	269	269	272	250

Table 12. *Activities of myokinase and adenylic deaminase in haemolysate*

(ADP, 10 μmol.; adenylic acid, 20 μmol.; adenylic deaminase (fraction 3*P*, Weil-Malherbe & Bone, 1951*b*), 1 mg./3 ml. Mineral constituents in standard concentration. Duration of experiment, 20 min.)

	Ammonia formation (μmol.)	
	From adenylic acid	From ADP
Fraction 3*P*	17·7	0·4
Haemolysate	10·6	—
Haemolysate + fraction 3*P*	—	3·2

leads to an increase of the reaction rate of brain hexokinase nor to a diminution of the activating effect of the haemolysate; ATP concentration was therefore not limiting even at the lower level, and under these conditions myokinase cannot be expected to affect the reaction rate. As will be shown in the following paper (Weil-Malherbe & Bone, 1951*b*), the addition of purified myokinase to brain extract does not cause any activation of hexokinase.

Since, in spite of the activation caused by the haemolysate, the relative rate of ATP and ADP utilization is hardly changed, it may be assumed that the mechanism of activation is the same in both cases. The fact that ADP utilization is capable of activation at all lends support to the suggestion (Weil-Malherbe & Bone, 1951 *a*) that the lowering of the reaction rate if ATP is replaced by ADP is not due to a

Table 13. *Utilization of ATP and ADP by brain hexokinase in presence of haemolysate*

(ATP, 10 μmol.; ADP, 20 μmol./3 ml. Activity of brain hexokinase with ATP = 100.)

	Hexokinase activity			
	With ATP		With ADP	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Brain extract	100	100	71	75
Brain extract + haemolysate	216	362	118	274

Table 14. *Activating effect of haemolysate in presence of excess ATP*

ATP added (μmol.)	Decrease in glucose (mg.)		Activating effect (activity of control = 100)
	Brain extract	Brain extract + haemolysate	
10	0·280	0·505	180
40	0·235	0·485	206

Table 15. *Formation of hexosediphosphate by brain extract in presence of haemolysate*

(Brain extract, 0·5 ml.; glucose, 11·1 μmol.; ATP, 20 μmol./6 ml. Duration of experiment, 20 min.)

Exp. no.	Addition	Δ Glucose (μmol.)	Δ HDP (μmol.)
1	Nil	−5·5	5·1
	Iodoacetate (1 mM)	−5·5	3·9
	Haemolysate	−9·6	5·1
	Haemolysate + iodoacetate (1 mM)	−8·8	8·5
2	Iodoacetate (2 mM)	−4·5	2·5
	Haemolysate + iodoacetate (2 mM)	−8·5	6·2
3	Nil	−3·6	2·6
	Haemolysate, dialysed	−5·6	4·1

Table 16. *Phosphate balance of phosphorylation of glucose by rat-brain extracts in presence of haemolysate*

(Brain extract, 0·9 ml.; glucose, 16·67 μmol.; ATP, 40 μmol.; haemolysate, 3 ml./10 ml. Duration of experiment, 20 min.)

Exp. no.	Δ Glucose (μmol.)	Activating effect (control = 100)	Δ Inorganic P (μmol.)	$\Delta P_{7\ min.}$ (μmol.)	P utilized (μmol.)	Δ ATP (μmol.)	ATP utilized (μmol.)	Δ ADP (μmol.)	P utilized / Δ Glucose
1	−14·2	201	3·9	−27·3	23·4	−14·8	12·8	2·2	1·65
2	−16·2	140	3·0	−33·7	30·7	−16·2	14·7	−1·2	1·90
3*	−11·7	162	1·3	−24·0	22·7	−12·0	11·3	0	1·94

* Dialysed haemolysate.

limitation imposed by myokinase activity, but to an inhibition by ADP. Were it otherwise one would have to assume a dual effect of the haemolysate, one on the hexokinase reaction and another on the myokinase reaction. It would be unlikely, in this case, that the difference in the relative rates of ATP and ADP utilization would remain constant.

This discussion, of course, is based on the assumption that animal hexokinases, like yeast hexokinase, are unable to utilize ADP directly as phosphate donor. Preliminary experiments with preparations of muscle hexokinase have indeed shown that the ability to react with ADP decreases with increasing purification.

Hexosediphosphate formation and phosphate balance. Tables 15 and 16 show that, as judged by the ratios of hexosediphosphate formation/glucose disappearance and P utilization/glucose disappearance, the reaction mechanism is not noticeably changed in the presence of dialysed or iodoacetate-poisoned haemolysate. Neither is there any significant increase in the formation of inorganic phosphate or ADP. These experiments, therefore, throw no further light on the mechanism of the activation effect.

DISCUSSION

The experiments described in this paper have established the existence of an activator of hexokinase in erythrocytes. The activator is a protein which is not identical with a number of enzymes known to be involved, directly or indirectly, in the process of glycolysis. No indication was found for the possibility that the activation was secondary to some changes in the reaction mechanism, such as an increase in the resynthesis or stability of ATP, or the removal of an inhibitory reaction product.

The activation effect appears to be due to a specific reaction, since the factor cannot be replaced by a number of unspecific proteins and since it only affects hexokinase. It is not our impression that the function of the factor is merely protective, since there was no appreciable falling off of hexokinase activity during experimental runs under the conditions used.

An identical or very similar principle has been found in muscle extracts (Weil-Malherbe & Bone, 1951*b*), and a more detailed discussion will be deferred until these experiments have been described.

SUMMARY

1. Red blood corpuscles of man and other species were found to contain a factor which increased hexokinase activity of rat-brain extracts and, to a smaller extent, the activity of rat-muscle hexokinase and of purified yeast hexokinase. In the majority of experiments the activity of brain hexokinase was about doubled in presence of the factor, but the highest effect observed was a sixfold increase of activity.

2. The effect on the phosphorylation of fructose by brain extracts was similar to that with glucose. The activity of phosphohexokinase, on the other hand, was unaffected.

3. The activator did not diffuse on dialysis and could not be separated from protein. It is not present in the stroma of the erythrocyte and is not identical with haemoglobin. It is comparatively heat stable, but loses its activity gradually when exposed to temperatures of 80–100°. Even after heating, the activity was associated with coagulated protein.

4. The activating effect cannot be adequately explained by the presence of hexokinase, phosphohexokinase, triosephosphate dehydrogenase or myokinase in the erythrocyte lysates. A study of the end products of the hexokinase reaction did not reveal any change of reaction mechanisms.

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Studies on Hexokinase

3. AN ACTIVATOR OF HEXOKINASE IN MUSCLE EXTRACTS

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It has been shown in the preceding paper (Weil-Malherbe & Bone, 1951*b*) that there exists in red blood cells an activator of the hexokinase reaction which could not be identified with any of several factors known to be parts of the glycolytic system. It will now be shown that a similar or identical factor is present in muscle. In view of the difficulties which were encountered in the attempts to purify the erythrocyte factor, emphasis in this study was laid on fractionation experiments. Fractions were obtained which showed maximal activity in concentrations of the order of 1 mg./3 ml.

METHODS

Most of the methods used in this investigation were fully described in the first two papers of this series (Weil-Malherbe & Bone, 1951*a*, *b*). Additional information is given below.

Measurement of the activating effect. To avoid any interference by muscle hexokinase all fractions which were to be tested for their activating properties were heated at 56° for 5 min., a treatment which practically destroys all hexokinase activity. No further mention will be made of this in the following sections, unless it is to be contrasted with other heat treatments.

The activating effect was measured by the increase in the rate of glucose disappearance caused by the addition of fractions of rabbit-muscle extract to rat-brain extract, and expressed in relation to the activity of the control experiment containing rat-brain extract only. The activity of the control experiment was designated by 100.

Measurement of phosphohexokinase activity. Phosphohexokinase activity was measured manometrically and/or by the estimation of hexosediphosphate formation. The test solution contained, in addition to the enzyme, the usual mineral constituents, adenosinetriphosphate (ATP) and hexosemonophosphate in the same concentrations as used for the estimation of hexokinase activity, unless otherwise stated.

Measurement of adenylic deaminase activity. The test solution contained $MgCl_2$ and $NaHCO_3$ in the usual concentrations and 10 or 20 μmol. adenylic acid/3 ml. Deamination was determined by the increase of NH_3 after incubation with the enzyme.

Measurement of adenosinetriphosphatase (ATP-ase) activity. The enzyme was determined by the formation of inorganic phosphate from ATP (10 μmol.) in a solution containing $MgCl_2$ and $NaHCO_3$ in the usual concentrations.

All experiments were carried out at 30° in an atmosphere of N_2 containing 5 % (v/v) CO_2 for periods of 10, 20 or 30 min. Estimations of metabolites (except glucose and fructose) were performed on trichloroacetic acid filtrates (final concentration 3–5 % w/v).

Protein was estimated with Folin & Ciocalteu's phenol reagent (1927).

Other experimental procedures are described in the following section.

RESULTS

Fractionation experiments

The starting material for further fractionation was an extract of chilled rabbit muscle prepared by homogenizing 1 vol. of tissue with 2 vol. of 0·033 M-$NaHCO_3$ solution in an Atomix blender (Measuring and Scientific Equipment Ltd., London, S.W. 1) for 2–3 min. The homogenate was gently stirred at 0° for 30 min. and strained through surgical lint. The extract was finally clarified by filtration through a layer of kieselguhr on a Büchner funnel. Further fractionation was carried out either by $(NH_4)_2SO_4$ addition or by isoelectric precipitation at pH 5·6.

Ammonium sulphate fractionation. The following fractions were collected: (1) 0–0·2, (2) 0·2–0·3, (3) 0·3–0·4, (4) 0·4–0·5 and (5) 0·5–1·0 saturation. The first four fractions were precipitated by saturated $(NH_4)_2SO_4$ solution adjusted to pH 7·6 with NH_3 and the last fraction by the addition of solid $(NH_4)_2SO_4$. The centrifuged fractions were washed once with $(NH_4)_2SO_4$ solutions of the requisite strength and dissolved in a small volume of 0·01 M-ammonium phosphate buffer (pH 7·6).

On dialysis in cellophan at 0° against distilled water (six to seven changes) fraction 1 formed an opalescent solution, whereas fractions 3–5 separated into a clear solution and a precipitate. Fraction 2 remained opalescent although a small precipitate was formed. The precipitates were centrifuged off, washed with distilled water and redissolved in phosphate buffer. These fractions were designated by the suffix *P*, the supernatant fractions by the suffix *S*.

In agreement with the results obtained by $(NH_4)_2SO_4$ fractionation of haemolysates (Weil-Malherbe & Bone, 1951*b*) it was found that the activator of hexokinase appeared in several fractions. It was strongest in fractions 1, 3*P* and 4*P*, less strong in fractions 2*P* and 5*P* and weak or absent in fractions 2*S*, 3*S*, 4*S* and 5*S*. This pattern, of which an example is given in Table 1, was repeatedly confirmed. The activation effect increases with increasing amounts of the factor up to a saturation value which, in the case of fraction 3*P*, was reached with the addition of about 1 mg. (Fig. 1). The maximum activation in this experiment was a little less than twofold, but higher effects were sometimes observed, especially when the amount of brain extract was reduced (Table 2). Fig. 2 illustrates the course of the reaction in presence and in absence of activating factor (fraction 3*P*) as measured by the disappearance of glucose.

Table 1. *Activating effects of ammonium sulphate fractions of rabbit-muscle extract on rat-brain hexokinase*

(1 mg. protein added to standard test.)

Fraction no.	Activating effect (activity of control experiment = 100)
1	196
2*S*	128
2*P*	166
3*S*	134
3*P*	226
4*S*	107
4*P*	209
5*S*	98
5*P*	132

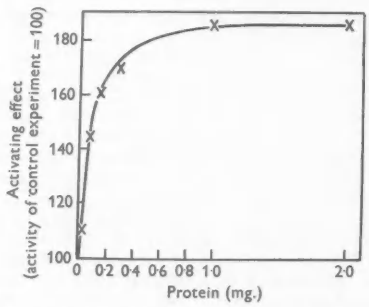

Fig. 1. Activating effect of fraction 3*P* on rat-brain hexokinase.

Table 2. *Activating effect of rabbit-muscle fraction 4P (1 mg.) on dilute brain hexokinase (0·5 mg. protein/3 ml.)*

	Decrease in glucose (mg.)	Activating effect (activity of control = 100)
Brain extract	0·080	—
Fraction 4*P*	0	—
Brain extract + fraction 4*P*	0·383	480

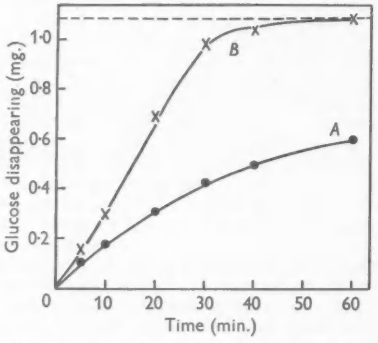

Fig. 2. Disappearance of glucose from rat-brain extract in presence and in absence of activating fraction 3*P* from rabbit muscle. Curve *A*, brain extract; curve *B*, brain extract + fraction 3*P* (1 mg. protein).

The fractions obtained by the addition of $(NH_4)_2SO_4$ were also tested for the presence of ATP-ase, adenylic deaminase and phosphohexokinase, and it appeared that their distribution closely followed that of the activating factor (Table 3).

Table 3. *Enzyme activities in ammonium sulphate fractions of rabbit-muscle extract*

Fraction no.	ATP-ase	Adenylic deaminase	Phospho-hexokinase
1	+ +	+ +	+
2	+ +	+ +	+
3*S*	0	0	(+)
3*P*	+	+ + +	+ + +
4*S*	0	0	0
4*P*	(+)	(+)	+ +
5*S*	0	0	0
5*P*	0	0	(+)

ATP-ase and adenylic deaminase activities were largely destroyed by heating at 56–60° for 5 min., but this treatment did not affect the activating factor and only moderately decreased phosphohexokinase activity. This last was completely abolished by heating at 80° for 5 min.; at this temperature the activating effect was also reduced to some extent (Table 4). The activating factor present in these muscle fractions thus seems to be less heat-resistant than the haemolysate factor. A further difference is that the activity of the heated sample is contained in the supernatant, and not in the coagulated mass of denatured protein as in the case of the haemolysate (Table 4).

Table 4. *Heat stability of activating factor*

(5 mg. protein added to standard test.)

	Activating effect (activity of control = 100)
Fraction 3*P*	
Heated at 56° for 5 min.	232
Heated at 80° for 5 min.	179
Heated with 0·05 vol. N-HCl at 90° for 2 min., cooled and neutralized	52
Fraction 4*P*	
Heated at 100° for 10 min., homogenized	146
Heated at 100° for 10 min., supernatant	139
Heated at 100° for 10 min., coagulum	99

Isoelectric precipitation. An active preparation was also obtained by acidification of the muscle extract to pH 5·6 with N-acetic acid. The resulting voluminous precipitate was centrifuged, washed with distilled water and dissolved by adjustment to pH 7·5 with 0·1 N-NaOH. A viscous, opalescent solution was thus obtained which had strong hexokinase, ATP-ase and adenylic deaminase activity, but contained only small amounts of phosphohexokinase. It was rich in activating factor, but somewhat higher amounts of protein, of the order of 5 mg., were necessary to reach the saturation value of the effect.

Attempts were made further to fractionate this material by salting-out techniques. By adding $(NH_4)_2SO_4$ solution to 0·2 saturation or Na_2SO_4 to 14% (w/v) a bulky precipitate was obtained which consisted largely of denatured protein. By further fractionation of the supernatant and subsequent

Table 5. *Heat stability of activating factor (isoelectric precipitate)*

(Samples heated in sodium phosphate buffer (0·01-M, pH 7·5) for 5 min.)

Protein added (mg.)	Temp. of heat treatment (°)	Coagulation	Fraction	Activating effect (activity of control = 100)
1	56	—	Whole	193
6	56	—	Whole	290
1	80	—	Whole	169
6	80	—	Whole	228
1	60	—	Whole	182
1	70	—	Whole	161
1	80	(+)	Whole	146
1	100	++	Whole	156
9	80	+	Whole	187
			Supernatant	197
			Coagulum	119
			Supernatant, protein-free*	104

* Precipitation with trichloroacetic acid, followed by ether extraction of filtrate.

dialysis, fractions were obtained which corresponded to fractions 2, 3*S*, 4*S*, 3*P*, and 4*P* of the preceding paragraph. Increasing the $(NH_4)_2SO_4$ concentration above 0·5 saturation did not produce any further precipitate. The activating factor showed a very similar distribution pattern, being present in the new fractions 3*P* and 4*P* especially, but the activity per mg. protein was not greatly increased compared with the original isoelectric precipitate. It must be assumed, therefore, that a considerable part of the factor was lost with the denatured protein.

The addition of acetone to the solution of the isoelectric precipitate at 0°, up to a final concentration of 30 %(v/v), led to the formation of a precipitate which, after dialysis, proved to contain activating factor together with hexokinase and phosphohexokinase. When this solution was further fractionated with $(NH_4)_2SO_4$, the bulk of the protein was again precipitated in denatured form by 0·2 saturation. With the remainder, fractions were obtained corresponding to nos. 1*S* to 4*S* and 1*P* to 4*P*. None of these had any hexokinase activity, but activation was caused by the fractions corresponding to 1*S*, 3*S*, 4*S*, 3*P* and 4*P*. The fractions showed maximum activity in amounts of 0·3–0·5 mg. protein.

Heat stability of the activating factor in the isoelectric-precipitate fraction. The activating factor in the isoelectric-precipitate fraction seemed to be less heat-sensitive than that in the fractions obtained by $(NH_4)_2SO_4$ fractionation (Table 5). Heating at 80° for 5 min. usually produced only an increase in opacity, but no outright coagulation. However, in a sample which had been kept at 0° for about 1 month after saturation with capryl alcohol (octan-2-ol), coagulation did occur leaving a clear supernatant. The latter contained the whole of the activity, while the coagulated protein was inactive. After the removal of protein from the supernatant with trichloroacetic acid, activity was lost (Table 5).

The greater heat resistance of the erythrocyte factor may perhaps be attributed to the formation of a bulky precipitate or to the liberation of SH-groups in denatured protein. Addition of egg albumin to the muscle fraction before heating did not, however, increase the heat stability of the factor, although massive coagulation took place. Addition of glutathione, either before or after heating, was likewise without effect. Finally, surface-active agents, such as capryl alcohol or 'cetavlon' (cetyltrimethylammonium bromide), added before or after heating of the muscle fraction, generally did not influence the activating effect except when added to a fresh preparation of the isoelectric-precipitate fraction (see below).

Increase of activating effect on standing. It was found that the activating effect of a solution of the isoelectric precipitate increased on standing, but that the full effect could be obtained in the freshly prepared fraction by saturation with capryl alcohol. This is demonstrated by the following experiment (Table 6): immediately after preparation part of the solution was sterilized by passage through a Seitz filter and put under aseptic precautions, into a number of small sterile bottles. Another part was saturated with capryl alcohol. Both fractions were kept at 0° and tested at various intervals. After 1 day the sterile fraction had only slight activity and only after 4 days was its activity equal to that of the capryl alcohol fraction. No capryl alcohol was added to the control since, according to Meyerhof & Wilson (1948), capryl alcohol inhibits brain hexokinase. Cetavlon had an effect on the activating factor similar to that of capryl alcohol.

Table 6. *Activation effect of isoelectric precipitate* (7 *mg.*)

(For details of procedure see text.)

Time after preparation (days)	Activating effect (activity of control = 100)	
	Sterile sample	Sample saturated with capryl alcohol
1	127	238
4	182	172
6	187	191
1	95	153
		Sample + 3·33 vol. 1 % (w/v) cetavlon 146

The hexokinase activity of the isoelectric precipitate fraction was at its maximum immediately after preparation and was stable for many days at 0°. It was not significantly affected by the addition of capryl alcohol or cetavlon.

Other muscle proteins. A preparation of rat myosin (Bailey, 1942) proved to be devoid of activating effects,

though it possessed strong ATP-ase and adenylic deaminase activities. The deaminase was separated from myosin by dialysis against 0·02 M-ammonium acetate (pH 8) according to Kalckar (1947). This fraction had no activating properties either. These results are of interest, since they confirm the conclusion, based on heating experiments, that the activating effect is independent of the activities of these enzymes.

Similarly, myokinase, prepared by the method of Colowick & Kalckar (1943), did not bring about any activation of brain hexokinase under the conditions of our experiments (Table 7). The preparation used was tested for activity

Table 7. *Effect of purified myokinase on the activity of brain hexokinase*

(For details see text.)

Myokinase protein added (mg.)	Activating effect (activity of control = 100)
0·05	96
0·5	100
1·0	97

in presence of adenylic deaminase (fraction 3*P*) and found to cause rapid NH_3 formation from adenosinediphosphate (ADP). Heating a preparation of activating factor (fraction 3*P*) with 0·05 vol. of N-HCl under the conditions used for the purification of myokinase completely destroyed its activity; the fraction even caused inhibition after this treatment (Table 4).

Effects of the activating factor of muscle

In all essentials the effects of the muscle factor resembled those of the erythrocyte factor. The presence of an active ATP-ase and adenylic deaminase in some preparations of the factor caused a larger formation of inorganic P and NH_3. The latter reaction leads to the formation of only about 1 mol. CO_2 per mol. glucose in manometric experiments, a fact which seemed puzzling until the correct explanation was found (Table 8). But the yield of hexosediphosphate (Table 8) and the utilization of $P_{7\,min.}$ (Table 9) per mol. glucose was the same, and the only significant difference in presence of the muscle factor was that the reaction went to completion more quickly. The factor, like that of erythrocytes, is specific for hexokinase and does not affect phosphohexokinase (Table 10).

Table 8. *Formation of carbon dioxide, ammonia and hexosediphosphate during glucose phosphorylation in brain extract containing activating factor of muscle (fraction 3P)*

(Brain extract, 0·6 ml.; glucose, 11·1 μmol.; ATP 20 μmol.; muscle fraction 3*P*, 2 mg./6 ml. Manometric reaction completed in 20 min. Control experiment (without muscle fraction) completed in approx. 60 min.)

CO_2 (μmol.)	ΔNH_3 (μmol.)	Δ Inorganic P (μmol.)	Δ HDP (μmol.)
10·90	12·0	3·0	8·2

Table 9. *Phosphate balance of glucose phosphorylation by brain extract in presence of activating factor of muscle (isoelectric precipitate)*

(Brain extract, 0·9 ml.; glucose, 16·67 μmol.; ATP, 40 μmol.; 21 mg. muscle protein/10 ml. Duration of experiment, 20 min.)

Δ Glucose (μmol.)	−14·0
Activating effect (activity of control = 100)	195
Δ Inorganic P (μmol.)	13·7
$\Delta P_{7\,min.}$ (μmol.)	−41·3
P utilized (μmol.)	27·6
Δ ATP (μmol.)	−20·6
ATP utilized (μmol.)	13·7
Δ ADP (μmol.)	0
$\frac{\text{P utilized}}{\Delta\text{glucose}}$	1·97

Table 10. *Effect of muscle fractions on activity of rat-brain phosphohexokinase*

(Hexosemonophosphate, 5·55 μmol.; ATP, 10 μmol./3 ml. Duration of experiment, 20 min.)

Addition		Activation of brain hexokinase (activity of control = 100)	Δ HDP (μmol.)	
Muscle fraction	Protein (mg.)		Without addition	With muscle fraction
Isoelectric precipitate (heated, 80°, 5 min.)	6	246	1·4	1·6
Isoelectric precipitate	6	171	1·8	1·8
Acetone precipitate	2	210	2·30	2·35

Table 11. *Effect of the activating factor of muscle on hexokinase preparations of various origins*

(Muscle extract and yeast hexokinase prepared and used as previously described (Weil-Malherbe & Bone, 1951*a*, *b*).)

Addition		Activating effect (activity of control = 100) on hexokinase of		
Muscle fraction	Protein (mg.)	Muscle	Brain	Yeast
Fraction 3*P*	5	138	199	—
Isoelectric precipitate	7	—	187	132
Isoelectric precipitate (heated, 80°, 5 min.)	7	—	156	146

The effect can be demonstrated not only with brain hexokinase, but also with hexokinase preparations of different origin and a higher degree of purity (Table 11), although it seems that it is highest when brain extracts are used. It is not restricted to the action of hexokinase on glucose, but may also be observed during the phosphorylation of fructose in brain extracts (Table 12). Fructose, as has recently been shown (Slein, Cori & Cori, 1950), is a substrate of brain hexokinase.

Table 12. *Effect of muscle fractions on phosphorylation of fructose by rat-brain extracts*

(Fructose, 5·55 μmol./3 ml. Fructose estimated by method of Nelson (1944).)

Addition		Activating effect (activity of control = 100) on phosphorylation of	
Muscle fraction	(mg.)	Glucose	Fructose
Isoelectric ppt.	7	278	206
Acetone ppt.	3·5	192	177

yeast hexokinase, which is not inhibited by hexosemonophosphate, is activated by the factor also argues against a connexion between the activating effect and the inhibition due to hexosemonophosphate. Adenylic acid, an inhibitor of myokinase (Colowick & Kalckar, 1943), has no effect on the activation of brain hexokinase by the muscle factor (Table 14).

Finally, as was previously found with erythrocyte factor, the activating factor does not increase the utilization of ADP in relation to that of ATP (Table 15).

DISCUSSION

Similar factors mentioned in the literature

Curtius & Ohlmeyer (1938) reported on the existence of heat-stable, non-dialysable factors in muscle extract which activated lactic acid formation from glycogen. They did not identify the step at which the activation occurred but, as hexokinase was not involved, it is unlikely that their observations have any bearing on the factor described in these communications. Curtius & Ohlmeyer saturated their system with every coenzyme of glycolysis known at the time. Since then the coenzyme of phosphoglucomutase, glucose-1:6-diphosphate (Leloir, Trucco, Cardini, Paladini & Caputto, 1948; Cardini, Paladini, Caputto, Leloir & Trucco, 1949) and of phosphoglyceric mutase, 2:3-diphosphoglyceric acid (Sutherland, Posternak & Cori, 1949) have been discovered and it is possible that the effects were due, at least in part, to the presence of one of these compounds.

Various other unidentified factors have been described as stimulating the glycolysis of Lebedew juice (Ohlmeyer, 1941, 1950) or of dried yeast cells (Meyerhof & Kaplan, 1950), but none of these has as yet been specifically assigned to hexokinase. Moreover, their diffusibility distinguishes them from the activating factor here described which in all probability is a protein.

Lipmann & Kaplan (1949) refer to a communication of Colowick (1947) which has not been accessible to us. Colowick is reported to have found in crude muscle extract a heat-stable, diffusible organic substance which stabilizes hexokinase, but which is apparently not an essential part of the hexokinase system. Were it not for its diffusibility this factor

Table 13. *Effect of activating factor (isoelectric precipitate, heated at 80° for 5 min.) on the inhibition of brain hexokinase by fructose-6-phosphate*

Fructose-6-phosphate (mM)	Brain extract: Decrease in glucose (mg.)	Brain extract: Inhibition (%)	Brain extract + muscle fraction (7 mg.): Decrease in glucose (mg.)	Brain extract + muscle fraction (7 mg.): Inhibition (%)	Brain extract + muscle fraction (7 mg.): Activating effect (control = 100)
0	0·288	—	0·450	—	156
0·55	0·195	32	0·210	53	—
1·85	0·020	93	0·082	82	—
5·55	0	100	0·010	98	—

Table 14. *Effect of adenylic acid on the activating effect of isoelectric precipitate from muscle on brain hexokinase*

(Isoelectric precipitate, 7 mg./3 ml.)

Adenylic acid added (μmol.)	Decrease in glucose (mg.)	
	Without factor	With factor
0	0·400	0·727
10	0·335	0·772

Table 15. *Effect of the activating factor of muscle (fraction 3P, 1 mg.) on the utilization of adenosinediphosphate (ADP) by brain hexokinase*

(ATP, 10 μmol.; ADP, 20 μmol./3 ml. Activity of brain hexokinase with ATP = 100.)

	Hexokinase activity with	
	ATP	ADP
Brain extract	100	75
Brain extract + muscle fraction	330	192

There is no indication for the hypothesis that the activation is due to the removal of an inhibitory reaction product. A preparation of the factor, free of phosphohexokinase activity, did not significantly relieve the inhibition of hexokinase by hexosemonophosphate, although it activated the uninhibited fraction of the enzyme (Table 13). The fact that

might well be identical with the activator here described.

Vestling, Mylroie, Irish & Grant (1950) recently found that a liver homogenate which had itself no hexokinase activity increased the activity of brain hexokinase. This observation suggests that the activator of hexokinase is present in liver.

The nature of the activator

The activating factor of muscle seems to be associated with a protein complex which also contains an active ATP-ase, adenylic deaminase, hexokinase and phosphohexokinase. The methods previously used for the preparation of these enzymes (Kielley & Meyerhof, 1948; Humphrey & Humphrey, 1950; Schmidt, 1928; Racker, 1947) are similar to those yielding the activating factor. According to Kielley & Meyerhof (1950) the soluble ATP-ase of muscle is a lipoprotein and it would be interesting to know if the same is true of other components of the complex.

In spite of their initial association the activating factor is independent of the activities of ATP-ase, adenylic deaminase, hexokinase or phosphohexokinase. This conclusion is based not only on the effects of heat treatment which, while destroying enzyme activities, does not greatly impair the function of the activating factor, but also on the lack of correlation in their occurrence which became apparent on further fractionation. Thus it was possible to obtain fractions containing the activator, but having little or no activity with respect to any one or more of these four enzymes, while others were devoid of activating effects, but possessed strong ATP-ase or adenylic deaminase activity. So far, however, whenever either hexokinase or phosphohexokinase activity was found, the activating factor was present as well. This raises the question whether the activator is identical with, or a fragment of, inactivated hexokinase or phosphohexokinase. Such a supposition practically amounts to assuming the existence of a prosthetic group or coenzyme, for which there is at present no evidence. Furthermore, when purified yeast hexokinase was inactivated by heating at 60° and added to brain extract, no activation was observed.

The mechanism of the activating effect

We know at present of only three participants in the hexokinase reaction, namely hexose, ATP and enzyme. The activating effect must be due to the interaction, directly or indirectly, of the activator with one of the three reaction partners. Reasons have been given (Weil-Malherbe & Bone, 1951*b*) why it must be considered unlikely that the activation of hexokinase can be explained by a resynthesis of ATP, due to myokinase action or another mechanism, and in this paper it is shown that purified myokinase itself has no activating effect under our conditions. An activation of a transphosphorylation mechanism is improbable for the further reason that the factor is specific for hexokinase and has no effect on phosphohexokinase.

An activation of hexoses, however, is well within the bounds of possibility. It has been shown by Gottschalk (1947; see also Slein *et al.* 1950) that the active form of fructose which is alone able to react with yeast hexokinase has the furanose structure. The suggestion has been made (cf. Bouckaert & de Duve, 1947) that in the case of glucose, too, it is the furanose and not the pyranose form which is phosphorylated. A catalyst may be envisaged which accelerates the establishment of the equilibrium between furanose and pyranose forms. We have tried to demonstrate a direct influence of the activator on the configuration of glucose by incubating glucose with a muscle-extract fraction. After deproteinization the pretreated glucose reacted with brain hexokinase at exactly the same rate as untreated glucose. This negative result does not, however, disprove the theory, since an effect can only be expected when the equilibrium is continuously disturbed by the removal of the furanose compound.

There is finally the possibility that the factor acts on the enzyme itself. In the first paper of this series it has been shown that unspecific protective agents, such as proteins and other colloids, —SH compounds, amino-acids and substances which form heavy-metal complexes did not activate brain hexokinase. The evidence reported in the second and third papers strongly suggests that activity resides in a highly active protein of specific properties. Its function is probably more than a mere stabilization of the enzyme, which showed no sign of rapid inactivation under our conditions; the effects which have been described should, therefore, not be confused with the unspecific protective effects of insulin and other proteins on crystalline yeast hexokinase in high dilution (Berger, Slein, Colowick & Cori, 1946).

The existence of a factor acting directly on the hexokinase molecule would be of particular interest in connexion with the problem of the hormonal control of hexokinase activity. Attempts to demonstrate hormonal influences on hexokinase have been less uniformly successful when tissue extracts, instead of intact tissues such as rat diaphragm, were used. In particular, no direct activating effect of insulin on hexokinase has been found in extracts, though such an effect must be postulated in view of the results obtained with diaphragms of hypophysectomized and hypophysectomized-adrenalectomized rats (Krahl & Park, 1948; Perlmutter & Greep, 1948; Bornstein & Nelson, 1948; Villee & Hastings, 1949). This discrepancy might be due to the intervention of other factors mediating between

insulin and hexokinase. The observations of Stadie, Haugaard, Marsh & Hills (1949) on the rapid reaction of insulin with a constituent of rat diaphragm point in this direction.

Further speculation seems useless in the present state of our knowledge, and the question whether the activator of hexokinase is concerned with the pyranose-furanose conversion or is a member of a hypothetical chain of factors connected with hormonal control must be left open.

SUMMARY

1. A non-diffusible activator of hexokinase, similar to or identical with that present in erythrocytes, was found in rabbit-muscle extracts. It was partially purified by fractionation with ammonium sulphate or by isoelectric precipitation at pH 5·6.

2. The activator is associated with an enzyme complex containing adenosinetriphosphatase, adenylic deaminase, hexokinase, and phosphohexokinase. Further fractionation showed, however, that the activator is not correlated with the activity of any particular one of these four enzymes. Moreover, they could be differentially inactivated by a degree of heating which did not materially impair the function of the activator.

3. On addition of increasing quantities of activator to rat-brain hexokinase the effect reaches a saturation value. With our more active preparations the addition of 0·5–1·0 mg./3 ml. was required for maximum effect.

4. The activator is fairly heat-stable, though less so than the erythrocyte factor. After coagulation the activity is contained in the supernatant from which it can be removed by deproteinization.

5. Purified myosin or myokinase had no activating effect on hexokinase.

6. The activator is specific for hexokinase, but does not affect phosphohexokinase. On the other hand, hexokinase activity is increased whether glucose or fructose is the substrate, whether the enzyme is derived from brain, muscle or yeast and whether it is crude or partially purified.

7. A study of the end products of the reaction offered no explanation for the activating effect. In particular, no indication was found that the effect was due to the removal of inhibitory reaction products.

8. The mechanism of the activation is discussed and two hypotheses are considered: (1) that the activator acts by catalysing the conversion of hexopyranose to hexofuranose; (2) that the activator acts directly on the hexokinase molecule, possibly as a member of a chain of factors mediating between hormones and enzyme.

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Cytochrome *c* Modified by Digestion with Proteolytic Enzymes

1. DIGESTION

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Although the importance of prosthetic groups to the activity of certain enzymes has been much studied, the part played by the protein portion of the molecule has received little attention. Haemoglobin, catalase and peroxidase all have the same prosthetic group, namely, protohaematin, yet the attachment of different proteins gives them distinct and characteristic properties. Thus haemoglobin is a respiratory carrier, capable of combining reversibly with oxygen without undergoing oxidation; catalase, an enzyme catalysing the decomposition of hydrogen peroxide and peroxidase, an enzyme utilizing hydrogen peroxide for the oxidation of a number of substances. Similarly, some flavoprotein enzymes, having the same prosthetic group, catalyse different reactions. In other words, the difference in their enzymic properties is a reflexion of the difference in the protein portion of the molecule.

It would seem interesting, therefore, to find out to what extent a modification in the protein portion of an enzyme which has a well defined prosthetic group would be reflected in the catalytic and other properties of the enzyme. For this type of experimental approach cytochrome *c* has the following advantages. It is stable within a wide range of pH and temperature, it is easily isolated and purified on a comparatively large scale and its biological activity can be estimated by simple methods.

MATERIALS

Cytochrome c

(1) *Cytochrome* c *with iron content of* 0·34%. This was prepared by the method of Keilin & Hartree (1945) from horse hearts. Fe content was usually 0·34%. In cases where a lower figure was found, it was always brought up to this value by diluting the solution to 1 l. and repeating the $(NH_4)_2SO_4$-trichloroacetic acid precipitation.

(2) *Cytochrome* c *with an iron content of* 0·43%. This was prepared from cytochrome *c* with Fe content of 0·34% according to the method of Keilin & Hartree up to the point of the filtration of the mixture of saturated $(NH_4)_2SO_4$ and cytochrome at pH 10. Instead of being dialysed, the filtrate was diluted with 80% saturated $(NH_4)_2SO_4$ to 1 l. and precipitated with 25 ml. 20% trichloroacetic acid. The precipitate was suspended in as little 0·05 N-NH_3 as possible and dialysed first against 0·005 N-NH_4 followed by 0·5% NaCl solution.

Provided that the starting material had Fe content of not less than 0·34% and a concentration of not less than 1% protein/ml., cytochrome *c* of Fe content of 0·43% was invariably obtained.

(3) *A simple method of preparation of cytochrome* c *of iron content* 0·43% *from cytochrome of iron content* 0·34%. Cytochrome *c* solution (12 ml.) (1%, Fe content, 0·34%) was placed in a 50 ml. centrifuge tube containing a few drops of $CHCl_3$. The solution was heated in a water bath so that the $CHCl_3$ boiled vigorously. The heating was continued until the precipitation of cytochrome *c* was virtually complete. The precipitate was centrifuged down, washed once with distilled water, and dissolved in 5 ml. of 0·1 N-NaOH. Exactly 5 ml. 0·1 N-HCl were then added and the solution kept in the refrigerator overnight. Next morning the light-brown precipitate was filtered off to give a clear filtrate containing cytochrome *c* (Fe content 0·43%) in 0·6% NaCl. The yield, measured spectrophotometrically, was usually 50–60%.

The cytochrome *c* thus obtained was found to have the same activity in the succinic oxidase and cytochrome oxidase systems as cytochrome *c* prepared by other methods. The Fe content was usually 0·43%, although occasionally preparations with slightly lower content (0·40–0·42%) were obtained.

Pepsin. This was crystallized from Hopkin & Williams pepsin by the method of Northrop (1946). The crystals obtained were collected on a filter, redissolved in water, dialysed until salt-free and freeze-dried to a colourless light powder. This material was kept *in vacuo* over $CaCl_2$ and retained its activity for over a year.

Trypsin and chymotrypsin. These were crystallized from fresh ox pancreas by the method of Kunitz & Northrop (1936) except that the conversion of trypsinogen to trypsin was carried out according to McDonald & Kunitz (1946). The enzymes were stored as described by these workers.

Papain. A commercial product of British Drug Houses Ltd. was used.

Heart-muscle preparation. This was prepared from horse heart according to Keilin & Hartree (1947) except that the following modification was introduced in the washing procedure. Minced heart muscle (350 g.) was placed in a muslin sac of about 4 l. capacity supported on an iron ring. A jet of tap water was then run into the sac at such a rate that the minced muscle was kept well stirred and the water level inside the sac was maintained about three-fifths full. Occasionally the running tap water was stopped, the water inside the sac was allowed to drain completely and the sac was squeezed hard to get rid of as much water as possible. After about 4 hr. washing and eight squeezings, the muscle pulp was usually free from myoglobin. It was then treated as described by Keilin & Hartree (1947). This preparation was always used within 5 days.

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Kidney preparation. This was prepared from horse kidney using essentially the same method as described by Slater (1949). This preparation was also used within 5 days.

METHODS

Cytochrome c. The Fe content of cytochrome *c* and of the autoxidizable cytochrome fractions was estimated according to the method of Keilin & Hartree (1945). The activity in the cytochrome and succinic oxidase systems was estimated as described by Slater (1949) except that in the cytochrome oxidase system 0·05 M-*p*-phenylenediamine was used as the substrate.

Proteolytic enzymes. The activity of the proteolytic enzymes was estimated by the method of Anson (1938) and expressed in haemoglobin units.

Spectrophotometric control of the course of digestion. The digestion of cytochrome *c* with the formation of an autoxidizable derivative was studied in the following way: portions of the digestion mixture were withdrawn at suitable intervals and mixed with a measured volume of 0·25 M-phosphate, pH 7·3. In the case of pepsin digestion, where the mixture contained 0·06 N-HCl, an equivalent amount of NaOH was also added to the portions withdrawn. The total amount of cytochrome *c*, autoxidizable and non-autoxidizable, was estimated by measuring the intensity of the absorption at 550 mμ. after reduction by $Na_2S_2O_4$. A current of pure CO was then passed through the solution and the absorption at 550 mμ. again measured. The molar concentration of autoxidizable cytochrome *c* is given by $(D_r - D_{co})/(\epsilon_r - \epsilon_{co})$, where D_r = density after reduction at 550 mμ., D_{co} = density after reduction and passing CO at 550 mμ., ϵ_r = molecular extinction coefficient of ferrocytochrome *c* at 550 mμ., $2{\cdot}8 \times 10^4$, and ϵ_{co} = molecular extinction coefficient of the CO compound of autoxidizable cytochrome *c* at 550 mμ., $1{\cdot}07 \times 10^4$ (Tsou, 1949).

The concentration of autoxidizable cytochrome *c* present can also be determined from the absorption at 550 mμ. of the reduced pigment before and after a current of air has been passed through the solution. Such results are less certain than those obtained by the CO method described above owing to the fact that aeration in presence of the reducing agent invariably leads to the formation of H_2O_2 which slowly oxidizes non-autoxidizable cytochrome *c*. Nevertheless, results were obtained which confirmed the general picture given by the CO method.

Isolation of a pepsin-modified cytochrome c

Digestion was carried out in 1 % cytochrome *c* solution to which was added a quarter of its volume of 0·3 N-hydrochloric acid and a measured amount (about 0·3 ml./100 ml. of cytochrome *c* solution) of 1 % pepsin solution, so that the amount of pepsin used was 0·15 haemoglobin unit per g. of cytochrome. The final pH of the solution was usually about pH 1·5–1·7 as measured with a glass electrode. The digestion was allowed to proceed for 16–20 hr. at 24° after which the reaction was stopped by the addition of an amount of N-sodium hydroxide equivalent to that of the acid initially added.

It was found that the component of the digest which showed the characteristic cytochrome *c* spectrum could be precipitated by either of the following methods: (1) by making the solution 85 % saturated with $(NH_4)_2SO_4$; (2) by freeing the solution almost completely of salt at pH 5. The procedure finally adopted for the isolation of this component was as follows. The neutralized solution was made 85 % saturated with $(NH_4)_2SO_4$, the mixture was cooled to 0° and filtered cold. The precipitate was dissolved from the filter using 1 l. of water per 2 mg. cytochrome *c*-Fe. The solution was then made 70 % saturated with $(NH_4)_2SO_4$ and 25 ml. of 20 % trichloroacetic acid were added per l. of solution. The precipitate was centrifuged down, suspended in as little 0·05 N-NH_3 as possible, transferred to a small cellophan tube and dialysed against 0·01 M-phthalate, pH 5. After three or four changes of the outer solution, the precipitation of modified cytochrome was almost complete. The contents of the cellophan tube were then rinsed with the same phthalate buffer into a centrifuge tube and the precipitate collected by centrifugation. The supernatant, still showing the cytochrome *c* type of spectrum, probably contained various breakdown products of cytochrome *c*. The precipitate, after being washed once with phthalate buffer, was dissolved in 0·02 N-NH_3 and the precipitations by $(NH_4)_2SO_4$, trichloroacetic acid and phthalate were repeated. After the second precipitation in the cellophan tube, the supernatant was almost colourless. The precipitate, washed once with phthalate buffer and once with a little distilled water, was dissolved in 2 % Na_2SO_4 to give a dark-red solution containing 3–4 mg. protein per ml. This solution was kept saturated with $CHCl_3$ at 0°. It contained a pepsin-modified cytochrome *c*, hereafter referred to as PM*c*.

Digestion with papain, trypsin and chymotrypsin

Conditions used in the digestion of cytochrome *c* with papain, trypsin and chymotrypsin are summarized in Table 1.

Separation of autoxidizable from non-autoxidizable cytochrome c. The mixture, containing about 50 μg. cytochrome-Fe per ml., was made 80 % saturated with $(NH_4)_2SO_4$, and left at 0° overnight. Next morning it was filtered cold (filtrate *A*).

Table 1. *Conditions used in the digestion of cytochrome* c (*cyt.* c) *by proteolytic enzymes*

(Concentration of cytochrome *c* in all cases, 1 % protein/ml. Fe content, 0·34 % except where specified in text.)

Proteolytic enzymes	Activator	pH	Buffer	Temp. (°)	Time of digestion (hr.)
Pepsin	—	1·5	—	24	20
Papain (50 mg./g. cyt. *c*)	—	4·9	0·1 M-Phthalate	4	72
Papain (50 mg./g. cyt. *c*)	Cyanide or cysteine	4·9	As above	24	20
Trypsin (0·05 Hb unit/g. cyt. *c*)	—	8·5	0·1 M-Borate	24	20
Chymotrypsin (0·05 Hb unit/g. cyt. *c*)	—	8·5	As above	24	20

The precipitate was then dissolved in water, reprecipitated by $(NH_4)_2SO_4$ and trichloroacetic acid and dialysed as usual to give a solution of autoxidizable cytochrome. Since the precipitation of autoxidizable cytochrome *c* in 80% saturated $(NH_4)_2SO_4$ is not always complete, the method can only be applied when a considerable proportion of the pigment is in the autoxidizable form.

Separation of non-autoxidizable from autoxidizable cytochrome c. Filtrate *A* was dialysed against 0·5% NaCl until free from $(NH_4)_2SO_4$ and then placed upon a chromatographic column prepared by mixing kieselguhr into a calcium phosphate gel until a suitable consistency was obtained. The intense red band was developed by alternate washings with 0·25 M-Na_2HPO_4 and water. A brownish-red substance was found to move down more rapidly than the main band which was red in colour. The brownish-red material on the column was rather diffuse, whereas the main red band was quite well defined. As soon as the two pigments were clearly separated, the column was cut through while still in the glass tube, the portion containing the main red band was eluted with sodium oxalate and precipitated from the eluate by means of $(NH_4)_2SO_4$ and trichloroacetic acid. The precipitate was finally resuspended in dilute NH_3 and dialysed as usual.

RESULTS

Digestion by pepsin

The change in the total cytochrome *c* concentration and the formation of an autoxidizable cytochrome is shown in Fig. 1. A curious result of this experiment was the fact that in presence of pepsin the total amount of cytochrome *c*, autoxidizable and non-autoxidizable, dropped to 60% in about 5 min. and then slowly returned to about 90% of the original amount in 1 hr. On the other hand, the amount of non-autoxidizable cytochrome decreased very rapidly so that in about 20–30 min. the cytochrome became completely autoxidizable. Similar results were obtained with cytochrome *c* preparations of both 0·34 and 0·43% iron.

The effects of temperature and of concentration of pepsin on the course of digestion were also examined. The results obtained made it clear that by varying temperature and pepsin concentration only the rate of the initial drop and the subsequent rise in the total concentration of cytochrome *c* were affected: the general shape of the curves remained unchanged. In all cases there was a quick initial decrease of the total cytochrome concentration to 60–65% of the original and a slow return to about 90%. The conversion of non-autoxidizable to autoxidizable cytochrome *c* was invariably complete as soon as the total cytochrome had passed the minimal point. The results obtained at 0, 20 and 38° are presented in Fig. 2.

With increasing pepsin concentration the time for reaching the minimal point was shortened, whereas the general shape of the curves remained unchanged.

Pepsin is very rapidly inactivated in neutral solutions; therefore, the digestion of cytochrome *c*

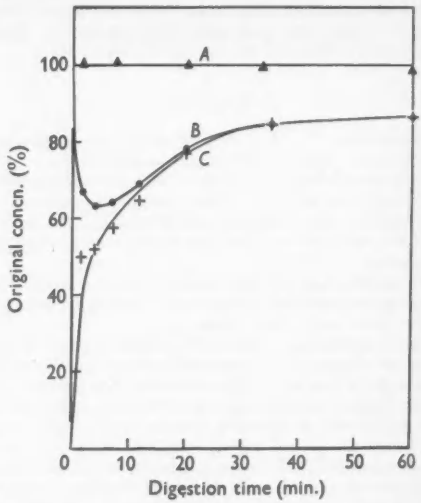

Fig. 1. The digestion of cytochrome *c* by pepsin showing the change in total cytochrome (autoxidizable + non-autoxidizable) concentration in terms of percentage of original concentration (curve *B*), and in autoxidizable cytochrome (curve *C*) against time (in min.) as compared with a control without pepsin (curve *A*). Temp., 38°, pH = 1·6, amount of pepsin = 0·15 Hb unit/g. of cytochrome *c*, original cytochrome *c* concentration = 0·4 mM.

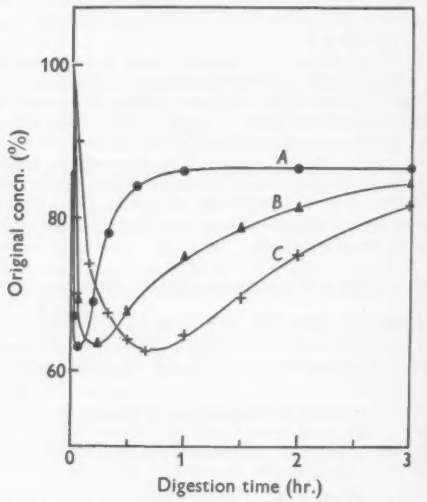

Fig. 2. The effect of temperature on the digestion of cytochrome *c* by pepsin showing the change in total cytochrome concentration (% of original concentration against time). Curve *A*, temp., 38°. Curve *B*, temp., 20° and curve *C*, temp., 0°. Amount of pepsin, pH, and original cytochrome concentration as in Fig. 1.

by pepsin can be stopped at any desired stage by the addition of alkali to bring the pH to neutrality. Making use of this property it was found that pepsin was responsible for the whole course of digestion. In other words, the presence of pepsin was necessary for both the initial drop and subsequent rise in the total amount of cytochrome *c*. A typical example to illustrate this point is shown in Fig. 3 in which the

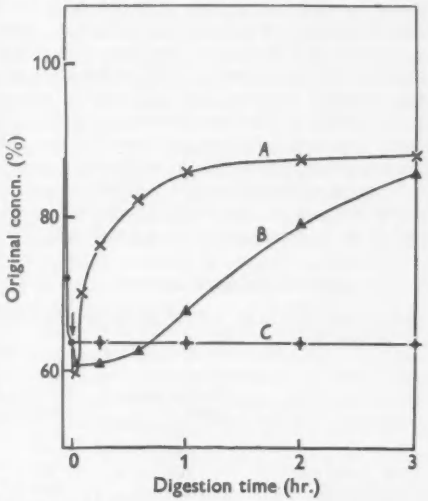

Fig. 3. Digestion of cytochrome *c* by pepsin interrupted by neutralization. Temperature, pH, amount of pepsin and original cytochrome concentration as in Fig. 1. The reaction was interrupted at the point indicated by the arrow. Curve *A* shows the change in total cytochrome concentration by re-acidification and addition of the same amount of pepsin; curve *B* shows a slow rise of total cytochrome concentration by re-acidification immediately after neutralization due probably to a partial re-activation of pepsin; curve *C* shows that the total cytochrome concentration remained unchanged if re-acidification was carried out 24 hr. after neutralization or if the reaction mixture was allowed to remain neutral.

digestion was stopped when the total amount of cytochrome *c* was very nearly at the minimum. If the reaction was interrupted at points other than the minimum, on re-acidification and addition of more pepsin the digestion always followed the same course it would have taken had the reaction not been interrupted by neutralization.

Digestion with papain

Digestion with papain in absence of activator. A pigment with the characteristic cytochrome *c* spectrum was isolated from the reaction mixture (Table 1, cytochrome *c* iron, 0·34 %) after the digestion and found to have an iron content of 0·48 % as measured by both the absorption at 550 mμ. of the reduced pigment or by the 2:2′-dipyridyl method. This pigment was non-autoxidizable and did not combine with carbon monoxide. It had approximately the same activity per iron atom as ordinary cytochrome *c* in the cytochrome oxidase system with either ascorbic acid or *p*-phenylenediamine as substrate, although if anything it slightly inhibited the succinic oxidase system of heart-muscle preparations.

Using the carbon monoxide method it was found that this pigment contained about 10 % autoxidizable cytochrome *c*. The non-autoxidizable fraction, when separated from the autoxidizable fraction, gave an iron content of 0·427 % and was indistinguishable from ordinary cytochrome *c* (iron, 0·43 %) in every respect. Thus the high iron content and the slight inhibitory effect of this pigment were due to the autoxidizable cytochrome present.

Digestion with papain in presence of an activator (Table 1). The pigment isolated in the usual way, by ammonium sulphate-trichloroacetic acid precipitation, at the end of the digestion contained 1·05 % iron. It was autoxidizable, combined with carbon monoxide in the reduced state and was biologically inactive.

Digestion with trypsin (*Table* 1)

At the end of digestion the reaction mixture was rapidly heated in a boiling-water bath to 95° and held at this temperature for 3 min. to destroy the trypsin activity. The solution was then filtered, diluted tenfold and the pigment isolated in the usual way by precipitation with ammonium sulphate-trichloroacetic acid. Cytochrome *c* thus modified, with an iron content of 0·87 %, was autoxidizable and combined with carbon monoxide.

Digestion with chymotrypsin (*Table* 1)

At the end of the digestion an estimation by the carbon monoxide method showed that the product was a mixture of autoxidizable and non-autoxidizable pigments. The solution was then heat-treated as above, filtered and the autoxidizable and non-autoxidizable fractions separated.

The non-autoxidizable fraction was found to have an iron content of 0·345 % and was indistinguishable from ordinary cytochrome *c* in every respect. If the starting material used was a cytochrome *c* preparation of iron content of 0·43 %, the non-autoxidizable fraction obtained also had the same iron content, 0·43 %. The autoxidizable fraction obtained had an iron content of 0·71 %. Like the other autoxidizable pigments obtained by pepsin, papain and trypsin, it also combined with carbon monoxide.

The course of digestions by papain, trypsin and chymotrypsin were also followed in respect of the total cytochrome concentration and the formation of

the autoxidizable fraction. The initial drop and the subsequent rise in total cytochrome *c* concentration, observed in the case of pepsin digestion has not been encountered in digestions by papain, trypsin or chymotrypsin. Moreover, after the initial drop in total cytochrome *c* concentration produced by pepsin digestion none of the proteolytic enzymes examined except pepsin could bring about the subsequent rise in the total cytochrome *c* concentration.

Activity of autoxidizable cytochrome c *in biological systems*

The autoxidizable fractions of cytochrome *c* modified by digestion with proteolytic enzymes were not reduced either by the succinic dehydrogenase system or by the dihydrocozymase-diaphorase system. They were not active in the succinic oxidase system nor in the cytochrome oxidase system with *p*-phenylenediamine as substrate. Details of the results obtained with PMc are shown in Table 2.

however, that cytochrome with 0·43 % iron is any 'purer' than the pigment with an iron content of 0·34 % as is believed by Theorell & Åkeson (1941). In any case, the question whether one of them is any purer than the other does not seem to be of much importance in view of the fact that neither of them is as active as the endogenous form (Keilin & Hartree, 1940, 1945).

In the light of the present investigation it is not difficult to understand that Altschul & Hogness (1938) observed only a very slight effect of carbon monoxide on the absorption spectrum of ferrocytochrome *c*. Keilin & Hartree (1939), using the manometric method, showed that although a concentrated solution of ferrocytochrome *c* took up some carbon monoxide, the amount was far less than could have been expected from a chemical combination between carbon monoxide and ferrocytochrome *c*. They attributed the slight effect obtained by Altschul & Hogness either to a small fraction of

Table 2. *Activity of pepsin-modified cytochrome* c (PMc) *in biological systems*

(O_2 uptake was measured at 38° in Barcroft differential manometers and expressed as Q_{O_2} (μl. oxygen uptake/mg. fat-free dry wt./hr.). Details of measurement were essentially the same as described by Slater, 1949.)

System	Components	Activity with: Heart-muscle preparation (Q_{O_2})	Activity with: Kidney preparation (Q_{O_2})
Cytochrome oxidase	Enzyme + *p*-phenylenediamine	590	250
	Enzyme + *p*-phenylenediamine + PMc, 0·055 mM	575	265
	Enzyme + *p*-phenylenediamine + cytochrome *c*, 0·055 mM	1540	635
Succinic oxidase	Enzyme + succinate	515	75
	Enzyme + succinate + PMc, 0·055 mM	460	60
	Enzyme + succinate + cytochrome *c*, 0·055 mM	665	215

DISCUSSION

By treating ordinary cytochrome *c* with N-potassium hydroxide for 18 hr. at 20°, Keilin & Hartree (1940) obtained an autoxidizable cytochrome *c* which was catalytically inactive in biological systems. They concluded that when cytochrome *c* becomes autoxidizable, it loses its catalytic activity. The findings of the present investigation amply confirm the conclusion reached by Keilin & Hartree.

All the autoxidizable cytochrome *c* fractions obtained in the present investigation have many properties in common. They are all biologically inactive, they combine with carbon monoxide in the reduced state and, moreover, they all have the same type of absorption spectrum which is very similar to that of ordinary cytochrome *c* in both the oxidized and reduced states (see Tsou, 1951). The results of these experiments seem to suggest that the protein portion of a cytochrome *c* molecule containing 0·43 % iron cannot be further reduced without affecting its catalytic activity. This does not necessarily mean, denatured cytochrome *c* or to some other haematin impurity. All the present autoxidizable cytochrome *c* fractions have been found to combine reversibly with carbon monoxide at all pH values and, moreover, the absorption spectrum of reduced autoxidizable cytochrome *c* has been found to be indistinguishable from that of the unmodified pigment. Consequently its presence in an ordinary cytochrome preparation could not be detected by the purity test devised by Altschul & Hogness. It is therefore likely that the cytochrome *c* preparation of these workers contained a fraction of autoxidizable cytochrome *c* whose carbon monoxide compound was responsible for the observed change in the absorption spectrum.

The findings of Paul (1948) that the loss of activity of cytochrome *c* in strong acid solution is not reflected in a corresponding decrease in the absorption at 550 mμ. of ferrocytochrome *c* can also be explained. The first stage of destruction of cytochrome *c* by acid is probably the slow transformation of cytochrome *c* into an autoxidizable derivative

which, although not biologically active, still has the same absorption spectrum as that of non-autoxidizable cytochrome *c*. By extraction with hot 0·1 N-acetic acid, Goddard (1944) obtained cytochrome *c* from wheat germ and found that it was less active than the pigment isolated from animal tissues. It is reasonable to assume that as a result of the drastic treatment his preparation contained a considerable amount of autoxidizable cytochrome *c* which would account for the low activity. It is also possible that the apparently high iron content of cytochrome *c* preparations obtained by Tint & Reiss (1950) was not due, as they believed, to any species differences but rather to the presence of an autoxidizable fraction.

The intensity of absorption at 550 mμ. of ferrocytochrome *c* has commonly been used as a measure of its concentration. However, the presence of an inactive autoxidizable fraction cannot be distinguished by this method. Although the amount of this autoxidizable material is usually negligible it can sometimes lead to a high iron content and thus create a false impression of the purity of the preparation. As a precaution it is therefore recommended, for estimation of cytochrome *c* concentrations by the spectrophotometric method, that a further set of readings be taken after a current of carbon monoxide has been bubbled through the solution. In this way not only the presence but also a rough estimation of the amount of autoxidizable cytochrome *c* present can be obtained.

SUMMARY

1. Digestion of cytochrome *c* by pepsin produced an autoxidizable pigment. During the digestion the total amount of cytochrome present, autoxidizable and non-autoxidizable, showed a rapid initial drop and a slow subsequent rise.

2. Digestion with papain in absence of an activator raised the iron content of cytochrome *c* from 0·34 to 0·43 % without affecting its catalytic activity. In presence of an activator papain produced an autoxidizable pigment.

3. Digestion with trypsin produced an autoxidizable pigment while chymotrypsin produced a mixture of autoxidizable and non-autoxidizable pigments. The non-autoxidizable part of this mixture, when isolated, contained 0·34 % iron.

4. None of the autoxidizable pigments was active in the succinic oxidase system or in the cytochrome oxidase system with *p*-phenylenediamine as substrate.

I wish to thank Prof. D. Keilin, F.R.S., for suggesting this problem and for his valuable advice and interest in this work.

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Cytochrome *c* Modified by Digestion with Proteolytic Enzymes

2. PROPERTIES OF PEPSIN-MODIFIED CYTOCHROME *c*

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In the preceding communication (Tsou, 1951) the digestion of cytochrome *c* by proteolytic enzymes and the isolation of autoxidizable fractions of cytochrome *c* thus modified have been described. These autoxidizable cytochrome *c* fractions have been shown to possess several properties in common, namely they are all autoxidizable, they combine with carbon monoxide in the ferrous state and they are not active in biological systems. The properties of one of them, that obtained by digestion with pepsin, will now be examined in greater detail. Some of the findings have already been reported briefly elsewhere (Tsou, 1949).

EXPERIMENTAL

Pepsin-modified cytochrome c (PMc). This was prepared as described (Tsou, 1951) and resisted further attempts at fractionation. The N and Fe contents of both the precipitate and supernatant were found to be unchanged (*a*) after part of the PMc had been precipitated at pH 6·5, and (*b*) after precipitating part of the protein at 50% saturation with $(NH_4)_2SO_4$.

Catalase. This was prepared according to the method of Keilin & Hartree (1937); the solution contained about 1% protein.

Ascorbic acid. Solution of desired strength was made up from ascorbic acid and neutralized with N-NaOH immediately before use.

Potassium cyanide. Solutions of desired strength were made up from A.R. KCN and neutralized with 0·3 N-HCl. These were kept in well stoppered bottles at 0° to minimize loss of HCN and used within a few days.

Methylcarbylamine. This was prepared according to the method of Gautier (1869) as modified by Hartley (1928). To ensure the complete removal of cyanide it was washed with dilute NaOH solution, saturated with NaCl, before the final fractional distillation.

β-Phenylhydroxylamine and nitrosobenzene. These were prepared as described by Gattermann (1943).

Cysteine. A solution of desired strength was prepared from cysteine hydrochloride neutralized immediately before use.

Gas mixtures. Mixtures of the required composition were made using the apparatus described by Hartree & Harpley (1949).

Buffers. The following buffer solutions were used for the pH ranges indicated: pH 3–5·5, phthalate; 6–8, phosphate (Sørensen); 8–10·5, borate.

Manometric experiments. These were carried out in Barcroft differential manometers at 38°. The substrate was added (by displacement of a dangling tube) at zero time after temperature equilibration. Readings were taken every 3 or 5 min. after the addition. Except when otherwise specified, the gas phase was air. In all cases the total liquid volume per manometer flask was 3·3 ml.

Spectrophotometric experiments were carried out in a Beckman spectrophotometer.

Amino N. Determinations made by the micro Van Slyke method (Peters & Van Slyke, 1932).

Paper chromatography. The method was essentially as given by Dent (1948) except that a mixture of *n*-butanol and acetic acid (Partridge, 1948) was used instead of collidine.

RESULTS

Properties of PMc

Analytical results. The contents of iron, total nitrogen, total amino nitrogen and α-amino nitrogen of PMc are listed in Table 1. For comparison, the values given for cytochrome *c* with 0·43% iron by Theorell & Åkeson (1941) are also given.

Table 1. *The iron, total nitrogen, total amino nitrogen, and α-amino nitrogen contents of* PMc *and ordinary cytochrome* c (Fe, 0·43%)

	Percentage contents of		No. of atoms/atom Fe	
	PMc	*c*, 0·43% Fe	PMc	*c*, 0·43% Fe
Iron	2·21	0·43	1	1
Total N	14·72	15·37	26·2	143
Total amino N	3·02	—	5·4	31–32
Total α-amino N	1·89	—	3·4	9–10

The molecular weight of PMc, calculated on the basis of its iron content, is about 2500, i.e. about 85% of the protein portion of the starting material had been removed by digestion.

Table 2. *The amino-acids (paper chromatography) present in* PMc *and in ordinary cytochrome c* (Fe, 0·43%)

Amino-acid	PMc	Ordinary cytochrome *c*
Alanine	+	+
Arginine	–	+
Aspartic acid	+	+
Cystine	+	+
Glutamic acid	+	+
Glycine	+	+
Histidine	+	+
Leucine	+	+
Lysine	+	+
Methionine	–	+
Phenylalanine	+	+
Proline	+	+
Threonine	+	+
Tryptophan	–(?)	+
Tyrosine	–	+
Valine	+	+

The amino-acids present in PMc and those of cytochrome *c* of iron content 0·43% were identified by the two-dimensional paper-chromatographic method. They are listed in Table 2. Cysteine is partially destroyed by acid hydrolysis, but it did show up on some of the papers. Tryptophan is destroyed by acid hydrolysis and hence cannot be detected by this method. PMc, however, gave a negative Hopkins-Cole test after alkaline hydrolysis indicating the absence of this amino-acid. In addition to tryptophan, tyrosine, arginine and methionine were also absent.

Absorption spectrum. The absorption spectrum of modified ferrocytochrome *c* (hereafter referred to as PMc^{++}) was identical with that of ferrocytochrome *c* in the visible region and in neutral and alkaline solutions. The absorption spectrum of pepsin-modified ferricytochrome *c* (PMc^{+++}) closely resembled that of types III and IV of ordinary ferricytochrome *c* as described by Theorell & Åkeson (1941). The absorption spectrum of PMc^{+++} in neutral solution has been described in a previous communication (Tsou, 1949).

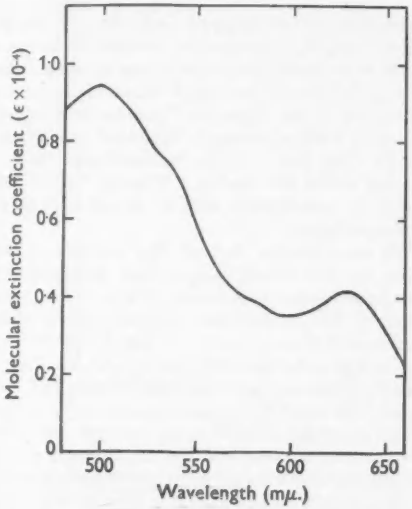

Fig. 1. Absorption spectrum of PMc^{+++} at pH 4·5. 0·1 M-Phthalate buffer; Na_2SO_4, 5%.

In acid solutions, with a pH of less than 4·5, the absorption spectrum of PMc^{+++} changed to a four-banded pattern very similar to that of type II of ordinary ferricytochrome *c*, i.e. of the acid methaemoglobin type. It showed a sharp, strong band at 630 mμ., weak bands at 580 and 535 mμ. and a strong, broad band centred around 500 mμ. This is shown in Fig. 1.

Haemochromogen-forming groups. Another interesting property of modified cytochrome *c* is that it contains no groupings able to combine with haem in N-sodium hydroxide to give a haemochromogen even after being heated for 10 min. at 95°. The formation of a haemochromogen with haem would be revealed by an extra α-band at 560 mμ.

Oxidation and reduction. PMc^{++} was readily oxidized by air, potassium ferricyanide and cupric salts. PMc^{+++} was easily reduced by sodium dithionite ($Na_2S_2O_4$) and BAL (2:3-dimercaptopropanol) at room temperature and by ascorbic acid at 38°. It was only slowly reduced by ascorbic acid and cysteine at room temperature, and not reduced by either the succinic dehydrogenase system or by the dihydrocozymase-diaphorase system.

Reaction with cyanide and methylcarbylamine. PMc^{+++} combined with cyanide and PMc^{++} with both cyanide and methylcarbylamine. The absorption spectrum of the PMc^{+++}-cyanide compound (shown in Fig. 2) is the same as that of ferricytochrome *c*-cyanide compound as described by Potter (1941) and Horecker & Kornberg (1946). It showed a broad band with its maximum at 537 mμ. Unlike the reaction between ferricytochrome *c* and cyanide, PMc^{+++} reacted with cyanide instantaneously.

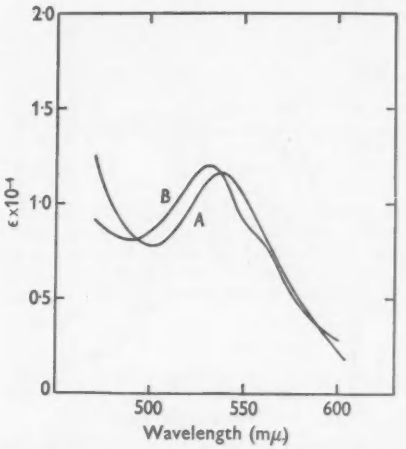

Fig. 2. Absorption spectrum of the PMc^{+++}-cyanide compound (*A*) as compared with that of PMc^{+++} (*B*). 0·1 M-Phosphate, pH 7·3; cyanide concentration, 5 mM.

The absorption spectra of the derivatives of PMc^{++} differed from that of PMc^{++} itself most markedly in the relative strength of the α- and β-bands. The absorption spectrum of the cyanide derivative has been given previously (Tsou, 1949), while that of the carbylamine derivative is shown in Fig. 3. The cyanide compound of PMc^{+++} was reduced with greater difficulty than PMc itself and the reduction of PMc^{+++} by BAL was found to be slowed down about ten times by 0·003 M-potassium cyanide. Similar results were also obtained with ascorbic acid as reducing agent.

Reaction with carbon monoxide. PMc^{++} combined with carbon monoxide showing two diffuse bands at 530 and 560 mμ., i.e. about 10 mμ. towards the shorter wavelength than the corresponding compound of haemoglobin. The absorption spectrum of this compound has been shown in a previous communication (Tsou, 1949). The carbon monoxide derivative of PMc^{++} showed slight light sensitivity

which could be demonstrated in the following way.

A solution of PMc in phosphate buffer, pH 7·3, was reduced by a little solid sodium dithionite and a current of CO was passed through. A strong solution of neutralized KCN was then added. During illumination for several minutes in a strong beam of light, the spectrum of the cyanide compound of PMc^{++} gradually replaced that of the CO compound. As soon as the light was turned off, the spectrum rapidly changed back to that of the CO compound.

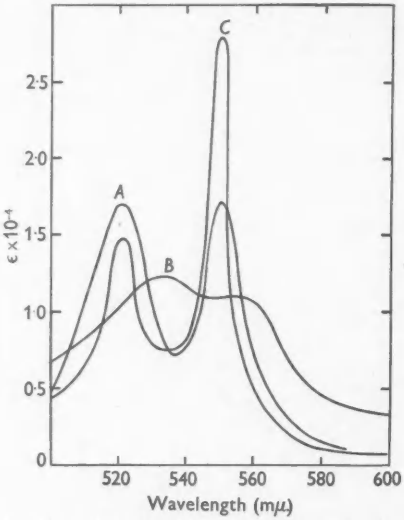

Fig. 3. Absorption spectra of the methylcarbylamine (*A*) and nitrosobenzene (*B*) compounds of PMc^{++} compared with that of PMc^{++} (*C*). 0·1 M-Phosphate, pH 7·3; methylcarbylamine, 5 mM; nitrosobenzene, 0·8 mM.

The carbon monoxide compound of PMc^{++} was oxidized by air, but less readily than the pigment itself.

Reaction with nitrosobenzene. PMc^{++} combined with nitrosobenzene forming a compound with an absorption spectrum closely similar to that of the carbon monoxide compound (Fig. 3).

Reaction with nitric oxide. PMc in both the oxidized and the reduced state combined with nitric oxide. The compound with PMc^{+++} had an absorption spectrum closely similar to that of the ferricytochrome *c*-NO compound, while the absorption spectrum of PMc^{++}-NO is of the type of the carbon monoxide and nitrosobenzene compounds. A comparison of the properties of PMc with those of ordinary cytochrome *c* at neutral and alkaline pH (types III and V) and with haemoglobin is given in Table 3.

Catalytic activity of PMc

On shaking a few drops of a solution of hydrogen peroxide with a PMc solution, a rapid evolution of oxygen was observed and the colour of the solution was rapidly changed to a light brown on account of destruction of the pigment. Cyanide, at a concentration of 0·002 M, strongly inhibited this decomposition. The Kat.f. (Euler & Josephson, 1927) of PMc was about 400 (Keilin & Hartree, 1937). PMc showed no peroxidatic activity at all with pyrogallol as substrate.

PMc was inactive in both the succinic oxidase system and the cytochrome oxidase system with *p*-phenylenediamine as substrate (Tsou, 1951). When tested in the cytochrome oxidase system using ascorbic acid as a secondary substrate, PMc showed, surprisingly, a pronounced activity. As PMc is rapidly oxidized by air, the presence of cytochrome oxidase was found to be quite unnecessary. In fact, in the catalysed oxidation of ascorbic acid, PMc acted not as an essential link in the cytochrome oxidase system but as an artificial ascorbic acid oxidase. This is clearly shown in Fig. 4. This oxidation of ascorbic acid appeared to be the most pronounced catalytic property of PMc, therefore it will be examined in greater detail.

Effect of 2:2′-dipyridyl on the reaction rate. The following experiments show that the catalytic effect of PMc on the rate of oxidation of ascorbic acid was due to the PMc itself and not to any free iron that might be present in, or produced from, PMc by destructive agents (e.g. hydrogen peroxide from autoxidation of ascorbic acid).

Table 3. *Comparison of the properties of* PMc *with those of ordinary cytochrome* c *in neutral* (c-*III*) *and alkaline* (c-*V*) *solutions and haemoglobin* (Hb)

	c-III	*c*-V	PMc	Hb
Autoxidizability	−	+	+	−
Reactivity of ferrous form with CO	−	+	+	+
Light sensitivity of CO compound	.	+	+	+
Reactivity of ferric form with cyanide	+	+	+	+
Reactivity of ferrous form with cyanide	−	+	+	−
Reactivity of ferrous form with carbylamine	−	+	+	−
Reactivity of ferrous form with nitrosobenzene	−	−	+	+
Reactivity of ferric form with nitric oxide	+	.	+	+
Reactivity of ferrous form with nitric oxide	−	.	+	+
Reaction with hydrogen peroxide	−	Destroyed with evolution of O_2		Oxidized
Haemochromogen-forming groups	+	+	−	+

In presence of ascorbic acid any free iron present would be in the ferrous state and would combine with 2:2′-dipyridyl to form a stable complex. It will be noted that saturation with 2:2′-dipyridyl did not affect the initial rate of oxygen uptake (Fig. 5) but that the rate fell off more rapidly in presence of 2:2′-dipyridyl. It will be shown later that hydrogen peroxide is produced during this oxidation of

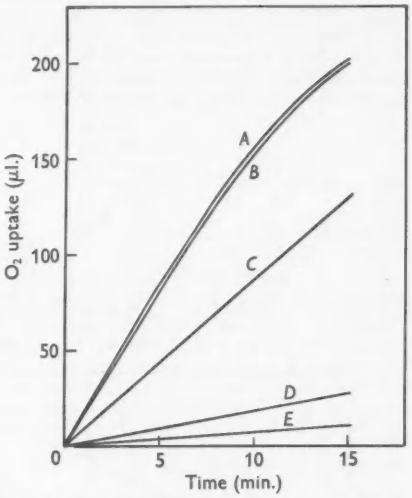

Fig. 4. Catalysed oxidation of ascorbic acid by PMc. 0·065 M-phosphate, pH 7·3; 0·025 M-ascorbic acid; temp., 38°. Curves *A* and *B* show the oxidation of ascorbic acid by PMc, 9·4 μM, in the presence and absence of a heart-muscle preparation (0·58 mg. fat-free dry weight) respectively. Curves *C* and *E* show the oxidation with ordinary cytochrome *c*, 9·4 μM, in the presence and absence of the same amount of heart-muscle preparation respectively. Curve *D* shows the autoxidation of ascorbic acid.

ascorbic acid, and it is therefore reasonable to conclude that the initial oxidation of ascorbic acid was due to PMc alone, whereas in the later stages of the reaction the iron set free from PMc by the action of hydrogen peroxide also contributed. By removing the ferrous ion as soon as it was formed, 2:2′-dipyridyl reduced the rate of oxidation of ascorbic acid in the later part of the reaction.

Coupled oxidation with catalase and ethanol. During the catalysed oxidation of ascorbic acid oxygen is reduced to hydrogen peroxide which can be demonstrated in a coupled oxidation with catalase and ethanol. This is shown in Fig. 6. It will be seen that the catalase partly protected the PMc from destruction by hydrogen peroxide so that the rate of oxygen uptake remained steadier in presence of catalase than in its absence. The addition of ethanol

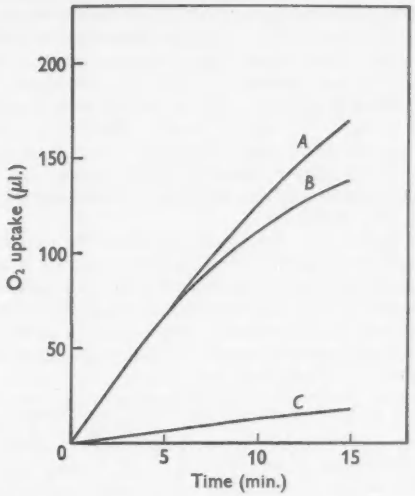

Fig. 5. Effect of 2:2′-dipyridyl on the PMc-catalysed oxidation of ascorbic acid. Conditions as in Fig. 4. Curves *A* and *B* show the oxidation catalysed by 7 μM-PMc in the absence and presence of 2:2′-dipyridyl respectively. Curve *C* shows control, autoxidation of ascorbic acid.

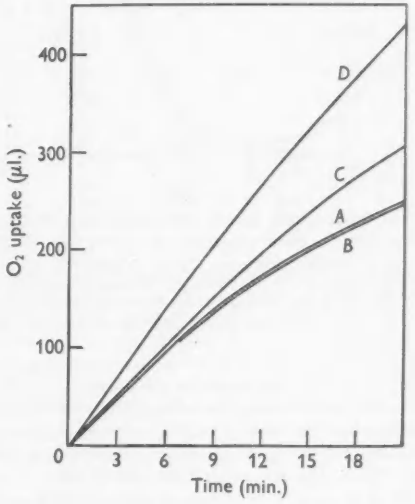

Fig. 6. Coupled oxidation with catalase and ethanol. Conditions as in Fig. 4, PMc, 9·4 μM. Curve *A*, PMc catalysed oxidation of ascorbic acid; curve *B*, as *A* in presence of ethanol; curve *C*, as *A* in presence of catalase; curve *D*, as *A* in presence of both catalase and ethanol.

alone, as was to be expected, produced no effect at all while the presence of both ethanol and catalase resulted in an increased rate of oxygen uptake due, no doubt, to utilization of hydrogen peroxide in the oxidation of ethanol. In this reaction ascorbic acid was oxidized far beyond the dehydroascorbic acid stage. In fact, about three to four times the theoretical oxygen uptake for the formation of dehydroascorbic acid was obtained and carbon dioxide was also produced.

Effect of pH *on the reaction rate.* The effect of pH on both the autoxidation and the PMc-catalysed oxidation of ascorbic acid is shown in Fig. 7. It must be pointed out here that the oxygen uptake obtained at alkaline pH was somewhat less certain owing to the rapid production of carbon dioxide which upset the pH of the buffered medium.

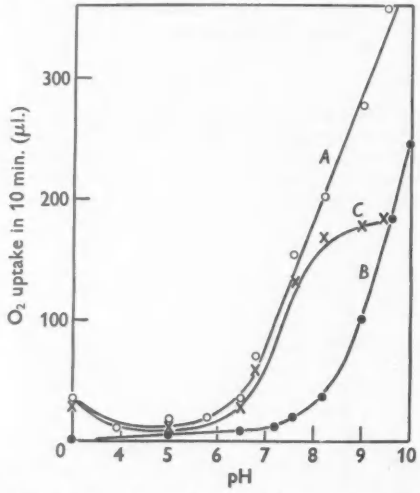

Fig. 7. Effect of pH on the PMc-catalysed oxidation of ascorbic acid. Conditions as in Fig. 4, PMc, 7 µM. Buffers: 0·1 M-phthalate, pH 3–6; 0·085 M-phosphate, pH 6–8; 0·1 M-borate, pH 8–10. Curve *A*, PMc-catalysed oxidation of ascorbic acid; curve *B*, blank showing autoxidation of same; curve *C*, PMc-catalysed oxidation of same corrected for blank.

Effect of PMc *concentration on reaction rate.* That the rate of oxidation of ascorbic acid is directly proportional to the amount of PMc present is shown in Table 4. The average value of Q_{O_2} (µl. oxygen uptake/mg. PMc/hr.) at 38° and pH 7·3 was 11,560.

Effect of ascorbic acid concentration on the reaction rate. This is shown in Fig. 8. It will be noted that the ascorbic acid concentration used in obtaining the Q_{O_2} (Table 4) was still far below the optimum.

Inhibition by carbon monoxide. It has been shown above that the reduction of PMc by ascorbic acid is somewhat accelerated in the presence of carbon monoxide. However, the reoxidation of the PMc-carbon monoxide compound by air is much more difficult than that of PMc itself. Manometric

Table 4. *Effect of* PMc *concentration on the rate of oxidation of ascorbic acid at* 38°

(Experiments carried out in Barcroft differential manometers. Each right-hand Barcroft flask contained 0·065 M-phosphate, pH 7·3; ascorbic acid, 0·025 M; 0·1 ml. of tenfold diluted catalase; and the concentration of PMc as specified in the table. KOH papers were used in the centre well. Q_{O_2} was calculated from the O_2 uptake in the first 10 min. and expressed as µl. O_2 uptake/mg. dry wt. of PMc/hr.)

PMc (µM)	O_2 uptake in first 10 min. (µl.)	O_2 uptake in 1st 10 min. corrected for blank (µl.)	Q_{O_2}
Nil	18	—	—
2·3	55	37	12,400
4·7	88	70	11,600
7·0	120	102	11,400
9·4	155	137	11,500
14·1	212	194	10,900
		Mean	**11,560**

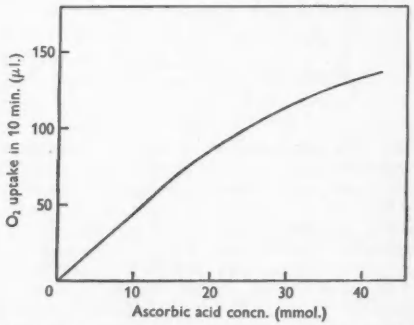

Fig. 8. Effect of ascorbic acid concentration on the PMc-catalysed oxidation of ascorbic acid. PMc, 7 µM; buffer and temperature as in Fig. 4.

measurements showed that carbon monoxide inhibited the catalysed oxidation of ascorbic acid by PMc to a considerable extent, e.g. in a mixture of 20% oxygen and 80% carbon monoxide, the reaction was inhibited by 85%. In agreement with the spectroscopic observations, the carbon monoxide inhibition was found to be somewhat light sensitive (Fig. 9).

The inhibition at different ratios of $CO:O_2$, but with a constant partial pressure of oxygen, was also estimated. The partition constant between oxygen and carbon monoxide of PMc was then calculated (Table 5). The calculations were made on the assumption that the carbon monoxide complex was completely inactive in the oxidation of ascorbic acid.

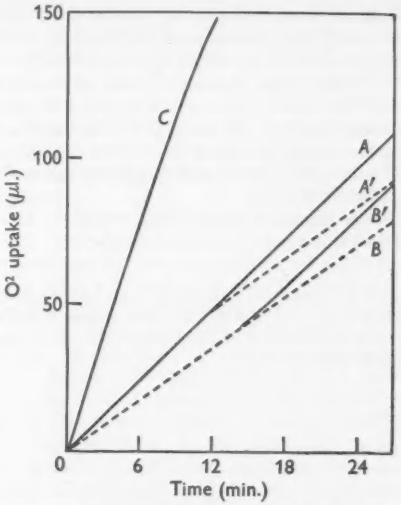

Fig. 9. Light dissociation of the PMc^{++}-CO complex. Conditions as in Fig. 4, PMc, 7 μM. Curve *C*, control in air; curves *A*, *A'*, *B* and *B'*, in 80% CO and 20% O_2. Curve *A*, reaction in light throughout; curve *A'*, reaction in light from start to 12th min., in dark from 12th to 27th min.; curve *B*, in dark throughout; curve *B'*, in dark from start to 12th min. and in light from 12th to 27th min.

Other inhibitors. Cyanide, methylcarbylamine, sodium nitrite and β-phenylhydroxylamine also inhibited this oxidation of ascorbic acid. A summary of the results is given in Table 6.

18 mM-Sodium azide, 18 mM-pyrophosphate and 0·8 mM-phenylthiourea had no effect on this reaction.

PMc also strongly catalysed the oxidation of dihydroxymaleic acid. It was ineffective in the oxidation of either cysteine or *p*-phenylenediamine.

DISCUSSION

The fact that the pepsin-modified cytochrome *c* has an absorption spectrum closely similar to that of ordinary cytochrome *c* seems to indicate that both the prosthetic group and that part of the cytochrome *c* protein which is in the immediate vicinity of the prosthetic group has not been appreciably changed during the digestion. The presence of cystine and histidine in PMc also supports this view if Theorell's (1941) hypothesis of the linkage of the prosthetic group to the protein is accepted. Chemical analysis points to the molecule of PMc consisting of three or four peptide chains containing approximately fifteen amino-acids. However, the chemical structure of the relatively small molecule of PMc, which may not be too difficult to elucidate, does not necessarily represent that part of the original cytochrome molecule which was in the immediate vicinity of the prosthetic group. The earlier claim of Wasteneys & Borsook (1924, 1925) that pepsin has synthetic as well as hydrolytic action does not seem to have been substantiated by later workers (Folley, 1932; Butler, Dodds, Phillips & Stephen, 1948), but nevertheless the possibility cannot yet be ruled out. The changes in the total concentration of cytochrome *c* during the pepsin digestion (cf. Tsou, 1951) give definite indication that some rearrangement within the molecule is taking place.

Most haematin compounds possess some oxidative, catalatic as well as peroxidatic, activity. As a haematin with a limited number of amino-acids attached, PMc is remarkable in that although

Table 5. *The partition constant of* PMc *between* CO *and* O_2

(Experimental conditions as in Table 4. PMc concentration, 8·4 μM. Partition constant calculated as

$$k=\frac{[PMc^{++}-CO]}{[PMc^{++}]}\frac{p_{O_2}}{p_{CO}}=\frac{I.R}{100-I},$$

where [PMc^{++}] and [PMc^{++} – CO] are the molar concentrations of PMc and its CO compound respectively; p_{O_2} and p_{CO} are the partial pressures of O_2 and CO respectively; *I* is the % inhibition and *R* the ratio of O_2 : CO.)

Composition of gas phase (%):				
O_2	20	20	20	20
N_2	80	70	50	0
CO	0	10	30	80
O_2 uptake first 10 min. (μl.), corrected for blank	113	72·6	39	17·5
Inhibition (*I*) (%)	—	35·8	65·5	84·5
Ratio, O_2 : CO (*R*)	—	2	0·67	0·25
Partition constant	—	1·11	1·21	1·36

Table 6. *Inhibition of the* PMc*-catalysed oxidation of ascorbic acid*

(Conditions as in Table 4. PMc concentration, 9·4 × 10^{-6} M. Temp. 38°.)

Inhibitor	Concentration (mM)	Inhibition (%)
Cyanide	0·8	57
	1·6	74
	3·2	87
Methylcarbylamine	1·5	46
	3·0	65
	6·0	93
Sodium nitrite	1·3	90
	2·6	100
β-Phenylhydroxylamine	0·4	48
	0·8	74

Most haematin compounds possess some oxidative, catalatic as well as peroxidatic, activity. As a haematin with a limited number of amino-acids attached, PMc is remarkable in that although

it has a high oxidative activity, it is very low in catalatic activity and is devoid of peroxidatic activity.

SUMMARY

1. The iron, total nitrogen, total amino-nitrogen and α-amino-nitrogen contents of PMc were determined and the amino-acids present were identified.

2. The adsorption spectra of the oxidized and reduced forms of this pigment resemble closely those of ordinary cytochrome *c*.

3. This pigment combines with cyanide, methylcarbylamine, carbon monoxide, nitric oxide and nitrosobenzene. It is autoxidizable and shows properties similar to type V of ordinary cytochrome *c* (i.e. cytochrome *c* at pH 13) on the one hand and haemoglobin on the other.

4. It is inactive in both the succinic oxidase system and the cytochrome oxidase system with *p*-phenylenediamine as substrate, but strongly catalyses the oxidation of ascorbic acid. As an artificial ascorbic acid oxidase, it has a Q_{O_2} of 11,500. This catalysed oxidation of ascorbic acid is inhibited by cyanide, methylcarbylamine, nitrosobenzene and carbon monoxide, the latter inhibition being somewhat light sensitive.

5. The pigment shows little catalatic activity and is devoid of peroxidatic activity.

I wish to thank Prof. D. Keilin, F.R.S., for suggesting this problem and for his valuable advice and interest in this work. Thanks are also due to Dr G. Wiltshire for the amino-nitrogen determinations.

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The Haemoglobins of the Human Foetus and Infant. Electrophoretic and Spectroscopic Differentiation of Adult and Foetal Types

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The differences between human adult and foetal haemoglobin have been summarized recently by Jope & O'Brien (1949) and by Kendrew (1949). Apart from the difference in the rate of denaturation by alkali, these differences were not sufficiently great to allow the determination of the small amounts of adult haemoglobin in the haemoglobin of the human foetus during early development and no such attempt has been reported. The statements on the relative amounts of the two types of haemoglobin at and after birth are not in good agreement. Most of the evidence is based on the difference in the resistance to denaturation by alkali, the foetal haemoglobin being the more resistant type. According to Haurowitz (1929, 1930) the blood of the newborn contains 80% resistant and 20% non-resistant haemoglobin. Jonxis (1949), at variance with a previous statement (Jonxis, 1948), reports that at birth the percentage of 'later' haemoglobin in healthy newborn infants ranges between 2 and 25% and that after 22 weeks all foetal haemoglobin disappears. Brinkman & Jonxis (1935) find that the foetal haemoglobin disappears entirely at 7 months after birth. The two types of haemoglobin in the newborn are immunologically different (Darrow, Nowakovsky & Austin, 1940). In the haemoglobin

of a 2·5 months premature infant a small amount of adult haemoglobin was present (titre with adult haemoglobin antiserum 1:16, with cord-haemoglobin antiserum 1:512). A difference in electrophoretic mobility between adult and foetal carboxyhaemoglobin was found by Andersch, Wilson & Menten (1944), but these authors studied cord blood only and they did not report the proportions of the two components.

In the present work the difference in mobility between adult and foetal oxyhaemoglobin was determined and the proportions of adult oxyhaemoglobin in foetal and post-natal red-cell extracts were studied by electrophoresis at low ionic strength, that is, under conditions resulting in large boundary anomalies. In addition, the ultraviolet absorption spectra were recorded. The position of the tryptophan fine structure band (the tryptophan 'notch') is 2898 A. for the foetal and 2910 A. for the adult haemoglobin (Jope, 1949) and it was anticipated that the spectra of mixtures of the two types might serve as a method of estimating their relative proportions.

EXPERIMENTAL

Preparation of oxyhaemoglobin solutions. Foetal samples were taken from the heart or from the umbilical cord, blood samples at birth from the placental end of the cord, those of the infant from the heel and those of the adult from the antecubital vein. The cells were washed with physiological saline, lysed with 2–4 vol. of distilled water and centrifuged. Addition of 0·1 vol. of 0·3 M-Na_2HPO_4 or of phosphate buffer, pH 7, to the supernatant brought down the remainder of the stroma. The solutions were put up for dialysis immediately. Samples of the concentrated solutions were diluted with 0·02 M-phosphate buffer, pH 7, to an appropriate concentration (about 0·02–0·03 g./100 ml.) and examined spectroscopically, usually without delay, or after storage in the refrigerator for not more than 2 hr.

Electrophoresis. The mobilities in phosphate at pH 7·1 and 8·0 were compared by the differential method (Hoch, 1949). The buffer at pH 7·1 was the same as used by Andersch *et al.* (1944) and at pH 8·0 an ionic strength of 0·05 was chosen.

All the experiments which served to analyse mixtures were made in 0·01 M-Na_2HPO_4 by the usual technique. The haemoglobin concentration was about 1 g./100 ml. After the ascending boundaries had migrated some three-quarters of the length of the compartment, the electrophoresis was continued while the boundaries were kept in position by compensation. The ascending boundaries only were observed. The descending boundaries were disturbed by convection. It has been pointed out (Hoch, 1950) that conditions resulting in pronounced boundary anomalies (low ratio of ionic strength:protein concentration) may be favourable for showing up small amounts of a faster component, the mobility of which differs little from that of the main component. These conditions apply to the analysis of foetal red-cell extracts which contain little only of the faster adult haemoglobin.

Evaluation of the electrophoretic patterns. Equation 1 (Hoch-Ligeti & Hoch, 1948) was used to calculate the corrected proportion of adult haemoglobin in the total and equation 2 to calculate the corrected relative difference in mobility.

$$C_1' = \frac{C_1'}{1 + \frac{V_1'}{S} K(C_t' - C_1')}, \qquad (1)$$

$$\frac{V_1' - V_2'}{V_1'} = \frac{S}{V_1'} \frac{C_1'}{C_t'} \frac{1}{1 + KC_t'}. \qquad (2)$$

(For significance of symbols see Hoch-Ligeti & Hoch (1948).)

These equations were derived on the assumption that the two components do not specifically interact with each other and that K is constant over the range of protein concentrations used. If the two components interact the corrected values for the proportion of adult haemoglobin represent minimum values and those for the relative difference in mobility maximum values. The neglect of the variation of K with the haemoglobin concentration is not of great consequence as can be judged from equations 1 and 2. For example, if K at 1·2 g./100 ml. were 0·135 instead of 0·123, the value for $C_1'/C_t' \times 100$ in the fifth analysis of Table 2 would be reduced from 25 by only 1·3. The haemoglobin concentration in the adjusted solution, C_t', was determined at the end of the run by the Kjeldahl method or colorimetrically. The coefficient K has previously been determined under identical experimental conditions to be 0·123 (Hoch, 1950) for adult oxyhaemoglobin at a concentration of 1·1 g./100 ml. and it was assumed that it is the same for foetal oxyhaemoglobin. The migration velocities were measured from the position of the original boundary and V_1' from $V_1' = V_{a1}'/(1 + KC_1')$ (Hoch-Ligeti & Hoch, 1948).

Spectroscopic methods. Logarithmic-cam moving-plate spectrograms were obtained with the improved version (Holiday, 1950) of the apparatus previously described (Holiday, 1937) by the same author. The wavelength of the tryptophan notch on each plate was determined by two or three observers, whose results did not normally differ by more than ±1 A. from the mean value.

The wavelength scale of the spectrograph was frequently checked by taking moving-plate spectrograms of a mercury resonance lamp, and using in particular the lines at 2803, 2894 and 2967 A. to check the scale in the 2900 A. region of the tryptophan notch. The wavelength scales of the actual haemoglobin spectrograms were also checked, using the hydrogen line at 3680 A., which occurs in the spectrum of the hot-cathode hydrogen arc used as the continuous light source.

A Unicam photoelectric spectrophotometer was used to obtain the data listed in Table 4.

Spectrograms of most of the samples in the ultraviolet were obtained with an automatic recording spectrophotometer to be described in detail elsewhere (Holiday & Sutton, 1951).

Stock solutions of adult and foetal oxyhaemoglobins for preparing the mixtures listed in Table 2 were made from fresh concentrated stock solutions by appropriate dilution with buffer as calculated from colorimetric analyses for oxyhaemoglobin, using the visible absorption bands, and the dilutions checked for equal haemoglobin concentration on the Unicam spectrophotometer.

RESULTS

Electrophoresis

Comparison of mobilities. In Table 1 are summarized the experimental conditions and the results of the differential electrophoresis. The adult oxyhaemoglobin was found to have a greater anodic mobility at both pH 7·1 and 8·0. The difference in mobility was higher at the higher pH.

least 6% adult haemoglobin. The pH in these experiments, which were made in 0·01 M-Na_2HPO_4, was about 8·8 and the difference in mobility between the two components was $0·3 \times 10^{-5}$ sq.cm. $V.^{-1}$ $sec.^{-1}$, which is greater than found at pH 8. Two full-term cord-blood samples were analysed at two concentrations of oxyhaemoglobin. The agreement in the results is taken as evidence that equations 1 and 2 are applicable.

Table 1. *Comparison of electrophoretic mobilities of adult and foetal oxyhaemoglobins by the differential method*

(In columns (2) and (8) A = adult, F = foetal; in column (4) asc. = ascending, desc. = descending. The minimum difference in mobility of components (column 7) is calculated from equation 2 of Hoch (1949).)

Exp. no.	pH of buffer	Haemoglobin concentration in top layer (g./100 ml.)	Potential gradient (V. $cm.^{-1}$)	Total change in distance (cm.) between the two ascending or the two descending boundaries	Total time (min.)	Difference in migration velocity of boundaries (10^{-5} cm. $sec.^{-1}$)	Minimum difference in mobility of components (10^{-5} sq.cm. $V.^{-1}$ $sec.^{-1}$)	Faster component
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
1	8·0	A 0·09 F 0·12	14	Asc. 0·47	320	2·4	0·17	A
2	7·1	A 0·22*	8·7	Asc. 0·10	140	1·2	0·14	A
3	7·1	A 0·5† F 0·5	8·7	Asc. 0·12 Desc. 0·17	300 300	0·7 0·9	0·09	A

* One experiment only with adult haemoglobin in top layer on ascending side.
† One experiment only with adult haemoglobin in top layer on ascending side and foetal haemoglobin in top layer on descending side.

Table 2. *Electrophoretic analyses of foetal and infant haemoglobin*

Source	Apparent proportion of faster component, % of total ($C_1'/C_t' \times 100$)	Apparent relative difference in velocity, % of V_{a1}' ($S/V_{a1}' \times 100$)	Haemoglobin concentration in adjusted solution (g./100 ml.)	Corrected proportion of faster component, % of total ($C_1'/C_t' \times 100$)	Corrected relative difference in mobility, % of V_1' ($(V_1' - V_2')/V_1' \times 100$)
(1)	(2)	(3)	(4)	(5)	(6)
Foetus, 20 weeks	26	2·2	0·72	6·5	8·7
Foetus, 20 weeks	34	1·7	0·86	6·9	7·9
Premature infant, 36 weeks	43	2·0	0·87	11	7·5
Full-term infant	55	3·0	0·96	21	7·6
Full-term infant, *a*	68	2·5	1·21	25	6·6
Full-term infant, *b*	39	3·1	0·65	15	7·5
Full-term infant, *a*	50	2·1	1·01	13	7·4
Full-term infant, *b*	36	2·6	0·74	16	8·5
Infants, 5 weeks (pool of two samples)	55	3·0	0·96	21	7·6
Infants, 2 months (pool of two samples)	79	4·8	0·88	54	6·8
Infants, 4 months	94	8·2	About 0·9	88	8·8

Foetal and infant haemoglobin. Fig. 1 and Table 2 show that there was a progressive increase in the relative area of the faster component as one passed from the 20-week foetus to 4-month infants, and that the correlated relative difference in mobility between the two components was constant over the whole range. The fast component was therefore assumed to represent adult oxyhaemoglobin in all cases. If this assumption is correct, then at 20 weeks the haemoglobin of the foetus already contained at

Artificial mixtures. To 20-week foetus oxyhaemoglobin different amounts of adult oxyhaemoglobin were added and the mixtures analysed by electrophoresis and spectrographically (Table 3 and Plate 4, Fig. 1*h*). It was found that the proportions of adult oxyhaemoglobin, corrected with the aid of equation 1, agreed better with those calculated from the amounts used in preparing the mixtures than the proportions as apparent from the patterns. However, the error in drawing the partition line between the two peaks

Table 3. *Electrophoretic analyses of artificial mixtures of adult and foetal haemoglobin*

Exp.	Proportion of adult haemoglobin added to 20-week foetus haemoglobin (%)	Haemoglobin concentration of adjusted solution (g./100 ml.)	Faster component found (%)		Faster component (%) calculated on basis of foetus haemoglobin containing	
			Apparent	Corrected	Apparent adult haemoglobin	Corrected adult haemoglobin
(1)	0	0·72	26·1	6·5	—	—
	22·4	1·05	57·1	22·4	42·7	27·4
	48·5	0·96	79·1	56·0	61·9	57·9
(2)	0	0·86	33·6	6·9	—	—
	34·0	0·90	64	35·1	56·2	38·6
	50·8	0·90	74	50·6	67·3	54·2

and in locating the boundaries appeared to result in an uncertainty of up to ±5 in the figure given for the corrected percentage of adult oxyhaemoglobin. Because a small percentage of adult haemoglobin produces an exaggerated boundary peak in the pattern of foetus haemoglobin, a foetal sample in which no adult haemoglobin is present would have been better suited for proving the validity of equation 1, but no such material was available for study, since the amount of blood yielded by 12-week foetuses was far from sufficient for electrophoresis and a foetus of an age of about 16 weeks, which possibly would have been free from adult haemoglobin, was not available.

Spectroscopic

In discussing spectrographic results, it is important to appreciate that the wavelengths found for the tryptophan notch are subject to error in the spectrograph wavelength scale and to plate-reading errors. In the period during which the present results were obtained, wavelength calibrations indicated the spectrograph scale to be in error by −1·5 to −3A. so that measurements on identical samples would be expected to vary over a range of 1·5A. With respect to reading errors, for three observers the range of disagreement for individual plates is not normally greater than ±1A. for the tryptophan notch. If both these sources of error operated together in the most unfavourable sense, identical samples would be expected to give readings differing by 3·5A. In practice, over short periods, measurements for example on normal adult samples, fall in the range 2909–2911A., that is a range of 2A., and it seems reasonable to assume that only wavelength differences greater than this are likely to be significant. The present discussion will be based on this assumption.

Considering first the samples of foetal origin which extend over the foetal age range 10–40 weeks, the latter being full-term cord bloods taken at birth, these all fall in the wavelength range 2897–2901A. with 2898A. as the most frequently observed value (mode) and 2898·3 as the mean (Table 4). The mode, 2898A., agrees with the figure given by Jope (1949) for foetal haemoglobin. Apart from the sources of error discussed above, it is of interest to note that the five samples giving 2897A. for the wavelength of the tryptophan notch all gave resolved maxima for this feature when examined on the automatic recording spectrophotometer at a spectral band width of about 7·5A., while the other foetal samples gave well

Table 4. *Wavelength of tryptophan notch in foetal and adult haemoglobin at* pH 7 *in* 0·02M-*phosphate buffer*

No. of samples	Description	λ (A.)
1	Foetal, 10 weeks	2898
2	Foetal, 12 weeks	2900
		2900
5	Foetal, 16 weeks	2898
		2898
		2898
		2897
		2898
2	Foetal, 20 weeks	2898
		(*a*) 2901*
		(*b*) 2900*
1	Foetal, 26 weeks	2898
6	Foetal, 36 weeks	2897
		2897
		2897
		2897
		2898
		2899
3	Full term, 40 weeks	2898
		2899
		2899
1	Infant, 2 months (pool of two samples)	2904
2	Infant, 4 months (pool of two samples)	2908
	Infant, 5 months (pool of two samples)	2905
12	Adult	2909
		2910
		2909
		2910
		2910
		2910
		2909
		2908
		2910
		2910
		2912
		2911

* Two separate preparations from whole blood.

defined inflexions, which were not resolved, although easy to distinguish from the poorly defined inflexions shown by adult samples. These differences are evident in Fig. 2. For foetal samples the difference between a well defined inflexion and a resolved maximum appears to be a question of instrumental performance, as it has been found that moving-plate spectrograms of foetal samples which did not give

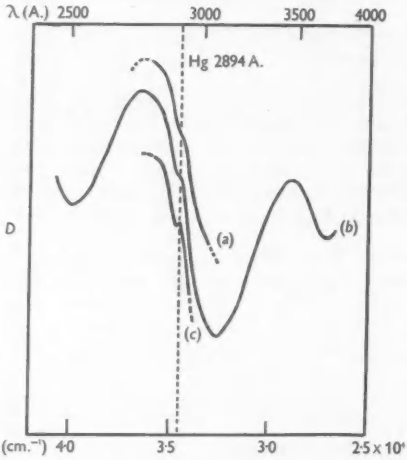

Fig. 2. Ultraviolet absorption spectra of adult and foetal human oxyhaemoglobins. (*a*) Adult, showing unresolved inflexion. (*b*) 36-Week foetal, showing unresolved inflexion. (*c*) 36-Week foetal, showing resolved tryptophan notch at 2897 A. The curves are direct tracings of spectrograms obtained with the automatic recording spectrophotometer at a constant spectral band width of 7·5 A. The curves correspond to approximately equal concentrations of oxyhaemoglobin, but for clarity have been displaced on the vertical co-ordinate which is linear in optical density (*D*).

resolved maxima in the automatic recording spectrophotometer, do show resolution of the notch in densitometer records of the plates (Plate 4, Fig. 3). In spite of this partial discrepancy between the automatic recorder and the moving-plate spectrograms, the densitometer records from plates of adult samples confirm the lack of resolution of the tryptophan notch in these samples and the relatively poorer definition of the resulting inflexion.

In the light of these comments, the wavelengths found for samples of differing foetal age cannot be regarded as showing any significant variation. As will be shown below, the position of the tryptophan notch in mixtures of foetal and adult haemoglobin is at, or very near, the foetal position (2898 ± 1 A.) if the proportion of adult type is not greater than 50 %.

The twelve adult samples all fall within the wavelength range 2908–2912 A.; the mode is 2910 A. and the mean 2909·8 A., in good agreement with the value (2910 A.) given by Jope (1949) for adult human haemoglobin. The range of values, extending over 4 A., is wider than would be expected from the sources of error discussed above, and arises from the single figures at the extreme of this range. One of these extreme values (2912 A.) was obtained at the same time that a 5-month foetal sample gave 2900 A., which is near the upper limit of the values for foetal haemoglobin, so that both these figures may be associated with a temporary instrumental error of +1 to +2 A., which later disappeared, as shown by the subsequent reversion to figures at or nearer the mean values for foetal and adult samples.

Table 5. *Wavelength of tryptophan notch in artificial mixtures of adult and 20-week foetal human oxyhaemoglobins*

Nominal composition*		
Foetal (%)	Adult (%)	λ (A.)
25	75	2905
50	50	2902–3
75	25	2901–2
50	50	2901–3
40	60	2902–5
30	70	2905–7
20	80	2906–8
10	90	2907–8

* Not corrected for 7 % adult type haemoglobin in 20-week foetal samples.

The spectroscopic results obtained with known mixtures of foetal and adult haemoglobins are shown in Table 5. For the mixtures containing 50 % adult type, the displacement of the tryptophan notch from the foetal position is barely significant if spectrograph errors of 1–2 A. have to be considered. For the mixture containing 10 % foetal and 90 % adult types the position is not experimentally distinguishable from the figure for the adult type itself. For the mixtures of intermediate composition the displacements from the foetal position are roughly consistent with their compositions, the positions of the notch moving away from the foetal to the adult position as the proportion of the latter is increased. From a graphical treatment of the results (Fig. 4), however, it appears that the position of the tryptophan notch is not a linear function of the relative proportions of the two forms, but that very little shift from the foetal position occurs until the proportion of adult type is nearly 50 %, so that almost the whole of the wavelength difference of some 12 A. between the foetal and adult types occurs in the composition range 50–90 % adult. It is not difficult to understand this behaviour when it is recalled that in the foetal type the tryptophan notch is much better resolved than in the adult type and will therefore tend to dominate the notch profile in

mixtures of the two types, so that relatively large proportions of the adult type will be required to influence the profile in mixtures.

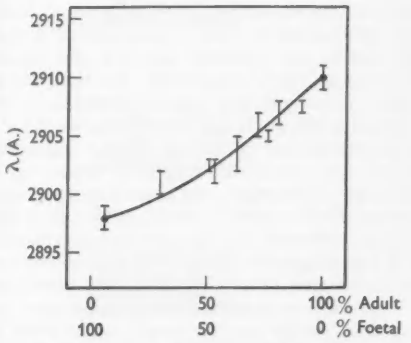

Fig. 4. Relation between the position of the tryptophan notch and the composition of artificial mixtures of adult and 20-week foetal human oxyhaemoglobins. The compositions have been corrected for the proportion (7%) of the adult type in 20-week foetal haemoglobin.

This dominant effect of foetal-type haemoglobin on the location of the tryptophan notch unfortunately precludes the use of this technique to check the estimates of adult type haemoglobin in foetal samples by the electrophoretic method (Table 2). As pointed out below, the two methods are in fair agreement in estimating the small proportions of foetal type haemoglobin in early infant blood samples. These are in the composition range in which the spectroscopic method is of definite value.

It may be noted at this point that in haemoglobin the tryptophan absorption is superimposed on that of β-phenylalanine and tyrosine, of which the latter makes an appreciable contribution to the total absorption of the globin moiety of the molecule. It is clearly of importance to the present work to ascertain if the superposition of an absorption band affects the observed wavelength of the fine structure. This situation has been studied by using mixtures of β-phenylalanine and diphenyl, in which the fine-structure bands of β-phenylalanine are superimposed on the smooth rising absorption of the longwave side of the absorption band of diphenyl. The results obtained by the moving-plate method are listed in Table 6 and show that over a wide range of relative contributions by these two compounds to the total absorption, the wavelengths of the β-phenylalanine fine-structure bands are constant to ±1A.

The intensities of the total aromatic amino-acid absorption bands in foetal and adult haemoglobins were compared by measuring the ratios of the densities of solutions of the two types adjusted to nearly equal concentration, at the wavelengths of the visible maxima and at selected points on the globin band. The results (Table 7) show that, relative to the haem absorption, the globin absorption is identical in the two types.

Some additional spectroscopic evidence is available regarding the state of the β-phenylalanine residues in foetal and adult human haemoglobins. The fine-structure bands of this aromatic amino-acid are clearly visible in moving-plate spectrograms of many proteins, including haemoglobin, and are found to be displaced to long wavelengths, in comparison with the free amino-acid, for reasons discussed below.

Table 6. *Wavelengths of β-phenylalanine fine-structure bands superimposed on diphenyl absorption band*

Solvent: 95% ethanol

(*R* = concentration of β-phenylalanine : concentration of diphenyl.)

	$\lambda_{max.}$ (A.)							
β-Phenylalanine alone	2365*	2419	2472	2523	2583	2613	2640	2678
β-Phenylalanine + diphenyl, *R* = 5·6	2368*	2418	2473	2522	2582	2613	2640	2678
β-Phenylalanine + diphenyl, *R* = 1·4	—	—	—	2522	2583	2613	2640	2678
β-Phenylalanine + diphenyl, *R* = 0·78	—	—	—	—	—	2612	2641	2678

* Very faint band.

Table 7. *Comparison of 20-week foetal* and adult haemoglobins in visible and ultraviolet light*

(Approx. 0·033% solutions in 0·02 M-phosphate buffer, pH 7; 1 cm. cells.)

λ (A.)	5760	5410	2900	2800	2700
$D^{1\,cm.}_{foetal}$	0·326	0·331	0·62	0·76	0·82
D_{5760}/D	(1·00)	1·05	0·525	0·43	0·40
$D^{1\,cm.}_{adult}$	0·336	0·316	0·62	0·77	0·85
D_{5760}/D	(1·00)	1·06	0·54	0·435	0·395

* Containing 7% of adult type haemoglobin.

Measurements of these band positions in foetal and adult human haemoglobins, which are reported in detail elsewhere (Beaven, Holiday & Jope, 1950), show that their positions are identical in the two types within the limits of experimental error, which range from ±2 to ±6A. depending on the band resolution.

DISCUSSION

From the experiments presented here it is apparent that the change from foetal to adult haemoglobin is a gradual process starting not later than midway

through pregnancy. In the earliest foetal sample examined (20 weeks) there is 6–7 % of adult type haemoglobin. At birth about 20 % adult haemoglobin was found, in agreement with the experiments of Haurowitz (1929, 1930). In infants 4 months of age only about 10 % foetal haemoglobin was present.

Adult and foetal haemoglobin dissociate into smaller molecules at low concentrations, but the two do not differ in the degree of dissociation (Gutfreund, 1949, quoted by Jope, 1949). In the present experiments dissociation must have been slow or suppressed since there was no evidence of the formation of mixed molecules with an intermediate mobility due to recombination of the fragments; two peaks only were seen in all instances.

The spectroscopic results for samples from infants of age 2 and 4 months respectively show progressive shifts from the foetal to the adult position of the tryptophan notch, and suggest that replacement of the foetal type of haemoglobin is very nearly complete 4 months after birth. It may be pointed out that this period is in good agreement with the average life of a red blood corpuscle as estimated by several concordant methods (Jope, 1946*a*, *b*). The proportion of adult-type haemoglobin 2 months after birth is 54 %, and 4 months after birth 88 %, by electrophoretic analysis (Table 2); from the measurements on synthetic mixtures of foetal and adult types the spectroscopic estimates of adult type in the 2- and 4-month infant samples are 60 % and about 80 %, respectively, in fair agreement with the electrophoresis figures. The rate of replacement of foetal by adult type haemoglobin after birth is thus of the same order when estimated by two independent methods.

The results given in Table 7 suggest that foetal and adult types of haemoglobin do not differ with respect to their total aromatic amino-acid composition. Porter & Sanger (1948) find by end-group analysis that the adult type contains 5 valine end groups compared with 2·6 for foetal type. As their sample of foetal type was taken from a 30-week foetus, with a possible adult type content of 10 %, the end-group analyses correspond more nearly to 2 valyl end groups in the pure foetal type.

The spectroscopic results confirm Jope's finding that the tryptophan fine-structure maximum occurs at a longer wavelength in adult-type human oxyhaemoglobin than in the foetal type, and further reveal that this shift is accompanied by a loss in resolution. For mixtures of the two types containing 50 % or more of the adult type, the position of the maximum can be used to estimate the proportions of the two types and the results are in fair agreement with the electrophoretic estimates of composition. No other differences in absorption spectra, either qualitative or quantitative, between the foetal and adult types of human oxyhaemoglobin, have been found.

Some comment on the difference in position of the tryptophan fine structure and in its degree of resolution in the foetal and adult types of human haemoglobin may be made on the basis of previous observations on synthetic peptides and proteins (Beaven *et al.* 1950). It is known that both shifts to longer wavelength and loss of resolution of fine structure occur in organic chromophores either by the introduction of non-absorbing substituent groups—the so-called weighting effect—or by interaction with polar, and especially hydroxylic, solvents (Brode, 1939; Morton, 1938). It appears unlikely, however, that the tryptophan residues in adult human globin contain such substituents and only environmental effects will be considered here. It is known from observations on tryptophan peptides that simple peptide combination shifts the tryptophan notch from 2880 A. in the free amino-acid to about 2892 A. with some loss of resolution, but this effect alone is inadequate to account for the shift to 2898 A. in the foetal type and to 2910 A. in adult-type human haemoglobins, or the conversion of the fine-structure maximum into a poorly defined inflexion in the latter type. In comparison with simple tryptophan peptides, the tryptophan notch may be shifted to 2910–2920 A. in some proteins, and it has been shown that this further shift can be observed in tryptophan peptides if these are examined in an environment in which strong solute-solvent interaction may be expected. A convenient protein medium for such observations is a dried gelatin film. The following results for glycyltryptophan are typical:

	λ_{notch} (A.)
Tryptophan (free acid in water)	2880
Glycyltryptophan (water)	2890
Glycyltryptophan (gelatin sol)	2890
Glycyltryptophan (gelatin film)	2917

Such an effect of a general protein environment on the absorption spectrum of tryptophan already combined in peptide linkage suggests that in adult haemoglobin a proportion of the tryptophan residues are oriented in a manner that leads to greater interaction with polar groups in the haemoglobin molecule itself or with water either as solvent or in the hydration layer, than for the foetal type. If this is indeed the case, the resulting additional longwave shift would tend to overlap and hence to obscure the resolved notch found in the foetal type, in which presumably the tryptophan residues are all subjected to sufficiently uniform environmental factors to give approximately equal longwave shifts and hence to show a resolved notch with a well defined maximum. A more detailed discussion of this suggestion would not be justified on the basis of present knowledge, but it is at least not inconsistent with the facts that

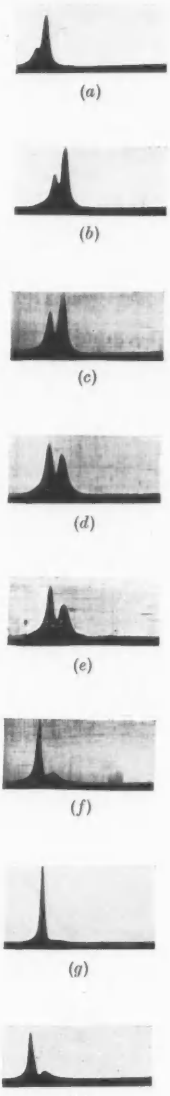

Fig. 1.

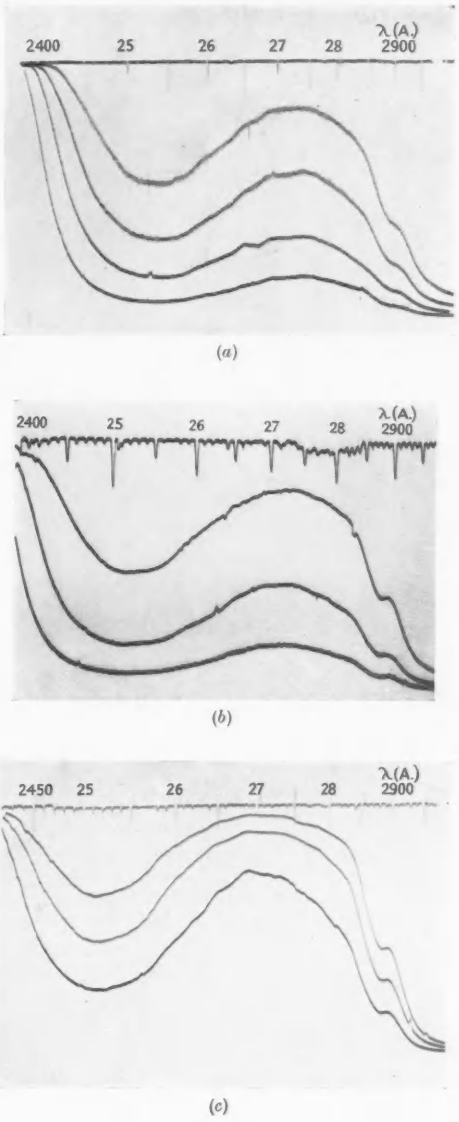

Fig. 3.

G. H. BEAVEN, H. HOCH AND E. R. HOLIDAY—THE HAEMOGLOBINS OF THE HUMAN FOETUS AND INFANT. ELECTROPHORETIC AND SPECTROSCOPIC DIFFERENTIATION OF ADULT AND FOETAL TYPES

the foetal and adult types also differ with respect to speed of spreading in monolayers and in rate of alkaline denaturation, both of which may be plausibly related to different configurations of hydrophilic and alkali-sensitive groupings respectively in the globin molecules of the two types.

SUMMARY

1. Foetal and adult haemoglobins have been studied by electrophoretic and spectrophotometric methods.

2. The differences in their properties when examined by these two methods have been used to follow their distribution in human blood from early prenatal life.

3. Adult oxyhaemoglobin shows greater anodic mobility than foetal oxyhaemoglobin in phosphate at pH 7·1, 8·0 and 8·8.

4. The findings of Jope on the differences in spectroscopic characteristics between the two types of haemoglobin have been confirmed and extended.

5. At 20 weeks of pregnancy foetal oxyhaemoglobin contains at least 6 % of 'adult' type; at birth about 20 %, at 2 months after birth about 50 %, and at 4 months between 80 and 90 % 'adult' type.

We wish to thank Prof. J. R. Marrack for his interest and encouragement; Dr E. A. Johnson for his contributions to the spectroscopic work; Dr J. C. White (Postgraduate Medical School) and Dr B. Levin (Queen Charlotte Hospital) and the Staff of the London Hospital Gynaecological Department for the supply of material.

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EXPLANATION OF PLATE

Fig. 1. Electrophoretic patterns of haemoglobins. (*a*) Foetus 20 weeks, 330 min. (*b*) Foetus 20 weeks, 325 min. (*c*) Premature baby 36 weeks, 337 min. (*d*) Baby full term, 307 min. (*e*) Babies 5 weeks, 303 min. (*f*) Babies 2 months, 217 min. (*g*) Baby 4 months, 119 min. (*h*) Artificial mixture, containing 58 % adult haemoglobin, 155 min. All experiments in 0·01 M-Na_2HPO_4 at 15·7 V. cm.$^{-1}$ Ascending patterns; the direction of migration is from right to left. The haemoglobin concentration can be read from Table 2.

Fig. 3. Microphotometer records of logarithmic-cam, moving-plate spectrograms of human oxyhaemoglobin solutions. (*a*) Adult, showing unresolved inflexion at 2911 A. (*b*) 20-Week foetal, showing resolved maximum at 2898 A. (*c*) 12-Week foetal, showing resolved maximum at 2900 A. All spectrograms taken on Hilger medium quartz spectrograph with slit of 0·15 mm. and hydrogen arc source, using Kodak B.10 plates. Samples (*b*) and (*c*) gave unresolved and resolved maxima respectively when examined on the automatic recording spectrophotometer at a spectral band width of 7·5 A.

Metabolic Response *in vitro* to Electrical Stimulation of Sections of Mammalian Brain

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Almost all metabolic studies of brain tissues *in vitro* have been carried out in ignorance of the electrical state of the separated tissue and without attempting to modify it. It is therefore difficult to assess the physiological significance of changes or lack of changes observed for example in respiration as a result of added substances, for the brain *in situ* exhibits continuous electrical activity. This activity can also be observed in small portions separated from certain parts of the frog brain (Libet & Gerard, 1939; Brooks, Ransmeier & Gerard, 1949), but does not appear to have been observed in the separated mammalian cerebral cortex (see de Barenne & McCulloch, 1941; Burns, 1950). In the whole animal, the electrical activity of the brain can be increased by electrical stimulation and increased metabolic activity accompanies this. More glucose and oxygen are taken from the blood stream and more carbon dioxide and lactic acid are added to it. The increased metabolic activity could thus support the greater expenditure of energy which the increased electrical activity can be supposed to involve. Changes in brain constituents can also be observed, notably in the energy-rich phosphates which also are probably involved in meeting the electrical requirements of the tissue. This subject has recently been reviewed by McIlwain (1950*a*).

The present studies were begun in order to see whether a similar relationship between metabolism and electrical changes which might act as stimuli could be detected in systems suitable for metabolic studies of brain tissue. (Electrical changes which might act as stimuli are referred to in the rest of the paper as 'stimuli'.) Most of the systems which have been used in the study of peripheral nerve were not suitable for this purpose (see below). Studies from these laboratories have already shown that sections of brain tissue under suitable metabolic conditions *in vitro* maintain or resynthesize the labile phosphates involved in functioning, a prerequisite for response to stimulation. If the normal relation between stimulation and metabolism is exhibited at the level of separated portions of the central nervous system, these preparations might respond to suitable electrical changes by increased respiration. We have found this to be the case.

Choice of experimental conditions

Study of the phenomena outlined above involves: (1) preparation of tissue; (2) application of electrical stimuli; (3) measurement of any metabolic response; (4) investigation of the effects of variation of the electrical stimuli and metabolic conditions. The present paper describes part of our studies of the first three of these, being concerned largely with the means of applying impulses to the separated cerebral cortex under conditions suitable for metabolic studies. To do this, we have selected the other conditions somewhat arbitrarily on the basis of existing knowledge or experimental convenience. The metabolic responses which have been measured may to some extent depend on these arbitrary choices and may not be optimal. The basis of choice was as follows.

(1) *Tissue.* The rates at which thin sections of cerebral cortex, cut parallel to the outer surface, metabolize glucose, can be very similar to those found for the brain *in vivo* (see especially Schmidt, Kety & Pennes, 1945). Such sections can resynthesize creatine phosphate and adenosine polyphosphates *in vitro* (McIlwain, Buchel & Cheshire, 1951; McIlwain, 1950*b*) and can maintain cozymase (Gore, Ibbott & McIlwain, 1950) although they contain enzymes which degrade these substances. They also maintain differential ion concentrations (Stern, Eggleston, Hems & Krebs, 1949; Terner, Eggleston & Krebs, 1950). Such sections were therefore chosen for the present work. The tissues are obviously to some extent damaged, and Libet & Gerard (1939) found comparable treatment reduced the spontaneous electrical activity of isolated frog brain. Nevertheless such sections are histologically relatively intact, and retain several relevant biochemical characteristics of organized structures.

(2) *Electrical stimulation.* Data are available concerning electrical, motor and other physiological responses to alternating and other forms of stimuli, applied to the cerebral or cerebellar cortex *in vivo* (see Schaefer, 1942). Most experiments have been made with cats, dogs, or primates without, however, showing marked differences with different species. Using sine-wave alternating current, Boynton & Hines (1933) found the optimal frequency for

responses at minimum voltage to be 60–90 cyc./sec.; motor responses were then found at 2–7 V. Faradic currents suitable for peripheral nerve were less suitable for the cerebral cortex. On the basis of such experiments, mains supplies of a.c. at 50 or 60 cyc./sec. have frequently been used for stimulating the brain (see, for example, Bailey & Sweet, 1940; Clark, 1939; Hines, 1940), and were therefore adopted for the present experiments.

Response to individual pulses has been found optimal with a full wave duration of 35–100 msec. and a rising phase of 6–15 msec. according to the system responding (Wyss & Alcade, 1937). For sustained activity at least 10 pulses/sec. were necessary. Rosenblueth & Cannon (1941–2) also found this to be the case for response at moderate voltage, and Moruzzi & Magoun (1949) report optimum response at minimum voltage to be given at 300 discharges/sec., and not to be given below 50/sec. This rate does not permit the individual pulses to be of the optimal duration quoted above, and the frequency used in the present experiments represents a compromise, being at 100/sec. Detailed study of the electrical characteristics of the stimuli required for optimum metabolic response does not however form part of this paper.

(3) *Measurement of metabolic response.* The manometric method has been chosen, since response to stimulation *in vitro* includes increased oxygen uptake and increased carbon dioxide and acid formation readily measured by this method. Two courses are open in devising suitable apparatus. One is to adapt apparatus which has been used for following gaseous exchange in peripheral nerve. Here, however, the rates of metabolism are lower than those of brain, and relatively little consideration has been given to exchange of metabolites between the tissue and a nutrient solution, and to their accurate determination. We chose the other course which was to use as basis the manometric apparatus in current biochemical use. This was used by Meyerhof & Schmitt (1929) in experiments with peripheral nerve, but does not appear to have been further developed. Our first type of vessel is shown in Fig. 3 and was made by sealing two electrodes through the sides of a conical Warburg vessel, whose centre well was displaced to allow for means of conducting current to a slice.

EXPERIMENTAL

General

Salines. Phosphate-glucose-saline contained 134 mM-NaCl; 5·4 mM-KCl; 1·34 mM-KH_2PO_4, $MgSO_4$, and $CaCl_2$; 13 mM-glucose; 10·4 mM-Na_2HPO_4 brought to pH 7·4 by HCl; it was saturated with O_2. Bicarbonate-saline contained 124 mM-NaCl; 5 mM-KCl, 1·24 mM-KH_2PO_4, $MgSO_4$ and $CaCl_2$; 12 mM-glucose and 26·1 mM-$NaHCO_3$, and was equilibrated with 5% CO_2 in O_2.

Brain tissue. Adult guinea pigs of about 400 g. and rats (Norwegian 'hooded') of about 300 g. were used. To obtain cortex slices as large and intact as possible, the following procedure was adopted. The animals were decapitated, the hair of the head was moistened with saline, the brain bared and the cerebrum removed undamaged with a spatula and put into a Petri dish which contained also filter papers moistened with saline. It was divided into the two hemispheres and these in turn placed, lateral cortex downwards, on a filter paper moist with the saline of the experiment, and resting on a suitable support. The lateral ventricle was entered with a small spatula, thin but not sharp. With this the cortex was unfolded into a sheet of tissue; its edges were nicked with the spatula at the anterior and posterior angles to facilitate this, and slight pressure was used. The basal ganglia still projected upwards, and were cut off by a sagittal cut in the plane of the ventricle with the spatula, leaving a sheet of cortex. The filter paper carrying the cortex was picked from its support, another moist paper put in its place, and the first with its cortex was put upside down on the second and then removed, leaving the cortex with its lateral face upwards. The cortex was inspected to ensure freedom from any fragments of bone or hair, and slices were cut with a strip of razor blading, using a recessed slide as guide. Before the blade was advanced in cutting, it was drawn backwards between the cortex and guide in order to sweep air bubbles from between these two. It was easy to obtain three successive large slices from a guinea pig hemisphere, and a fourth which was sometimes less perfect.

The slices were floated from the blade or guide to a dish of the experimental saline. If necessary they were trimmed to sizes to fit the various tissue-holding electrodes described later. This was done by pressing them against the bottom of the dish by a spatula blade (of plastic or stainless steel; about 12 mm. across and rounded). They were picked from the saline by the tared wire carrier of a torsion balance, drained on a glass surface, and the carrier and slice reweighed. With the carrier they were returned to the saline and opened by shaking the carrier and sometimes also with the help of a small camel-hair brush wet with saline. The slices were then ready for fitting to electrodes as is described below.

The dry weight of slices handled in this way was found to be 0·141 of their wet weight. This is a mean ratio derived from values in twelve experiments, some of which concerned the first slice cut ('outer') and others the subsequent slices ('inner'). No difference was found between outer and inner slices.

Manometric measurements. In general the ordinary techniques were employed, using constant-volume manometers with 300 mm. scales. Much of the apparatus was specially devised for the present work and is described below. Vessels and manometers were of Pyrex glass. The general experimental arrangement is shown in Fig. 1.

Electrodes. The platinum wire used was of 26 s.w.g. (0·457 mm. diam.) except when otherwise stated, and was flattened at the points at which it was sealed through the glass, to avoid loosening on cooling. The tungsten electrodes were of wire of the same size, and after sealing were cleaned electrolytically. Gold plating on platinum was from a solution made from gold chloride (equivalent to 0·34 g. Au) and KCN (1·9 g.) in 100 ml. of water. The electrode vessel was filled with this. Plating was at 70° with a current density of 10 ma./sq.cm. or a little lower, from a 2 V. accumulator with an ammeter in series. Both electrodes in a vessel were plated together by making them cathodes; it was not possible to plate one alone as any gold, already on the other, dissolved in the cyanide mixture in absence of an adequate applied

potential. The current flowing in plating was adjusted by the extent to which the anode, a heavy gold wire, dipped to the solution. Gold, melting some 700° below platinum, could not itself be sealed to the glass vessels.

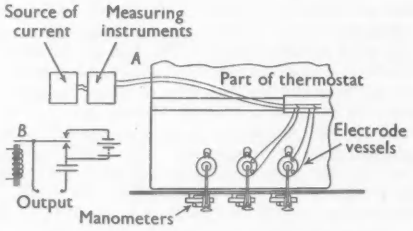

Fig. 1. *A*, plan of apparatus (semi-diagrammatic); *B*, circuit used for stimulating by condenser pulses.

Calibration of manometric apparatus. This involved determining the volume V of each vessel with its manometer to a fixed calibration point, and also that of the tissue-holding electrodes. The vessels and manometers were usually calibrated with Hg by the normal methods. No difficulty was presented by Pt or W lead-in wires. It was necessary, however, that plating with Au should follow the calibration, as Au amalgamates extremely easily. Occasional recalibration of vessels with gilded electrodes was done with water, but it was more satisfactory to remove the Au, use Hg, and replate.

For the tissue-holding electrodes, a different method was necessary. The volumes of small pieces of metal wire and gauze, when used alone, were ignored. When mounted in plastic holders, the volume of the whole—plastic, wire and any gauze—was determined by Archimedes principle. The electrode was suspended by a fine wire (stainless steel, 48 s.w.g.; 0·0406 mm. diam., was convenient) from a balance arm and weighed in and out of water at a known temperature. It was important to remove air bubbles from the apparatus, and a small camel-hair brush was used for this.

The volume of the electrode was then subtracted from V to give the volume, V_E, of the empty vessel, used for calculating vessel constants.

Electrical stimulation. (*a*) Sine-wave a.c. was taken from mains supply (50 cyc./sec.) through either an ordinary transformer or a 'Berco Regavolt' model 1A, which gave continuous variation in voltage although it was used at the end of its range. The ordinary transformer was arranged with about twelve steps between 1 and 5 V., and three input ranges so that a variety of output voltages was available. These and the currents were measured with a moving-coil meter and accordingly are mean and not peak values. Findings which illustrate the electrical characters of the cells used are given below. Both transformers could deliver at least 0·5 amp. without any fall in voltage.

(*b*) Condenser pulses were obtained from a circuit (Fig. 1, *B*) containing a variable capacity in series with the tissue-holding electrodes. A vibrator alternately charged the condenser from a battery, through the electrodes, and discharged it through them. The pulses thus had no d.c. component. Their characteristics were measured with a cathode-ray oscillograph.

Design of apparatus

Choice of materials for electrodes and lead-in wires. Conducting materials were required which did not seriously change or cause serious changes when carrying a.c. currents of up to 100 ma. at a few volts through typical metabolic solutions, nor when carrying brief impulses up to 20 V. For lead-in wires the material must be sealed through glass and cleaned with the glassware. Conditions used for stimulating peripheral nerve have in general made less exacting demands than this on an electrode material, as the experiments have not involved passing such currents through some ml. of nutrient solution. The vessels used for examining electrode materials were similar to (*A*), Fig. 3, with lead-in wires fused high in their walls, so that liquid media did not reach the leads themselves. To the leads were attached the materials to be examined, so that these materials were partly immersed in the liquid media.

Platinum was the least suitable of all electrode materials examined. It catalysed an appreciable oxidation of glucose and of some amino-acids in the salines described above shaken at 37° in air or oxygen. Ordinary bright platinum wire was effective in doing this, and did not change in appearance during reaction. Thus, about 1 sq.mm. of platinum as wire 0·06 mm. diameter, when conducting an alternating current of 8 ma. at 3 V. through glucose-phosphate-saline caused absorption of about 10 μmol. O_2/hr. During a similar experiment in bicarbonate-glucose-saline, evolution of gas occurred. Presumably an acid is formed by the catalytic oxidation of glucose.

Gold and silver formed suitable electrodes. No gas exchange was observed with the media and currents described above, nor when much greater areas and currents were used. The metals, however, underwent a surface change where they were in contact with the salines, giving under ordinary daylight conditions a black, closely adherent film, presumably initiated at least in the case of silver by the 'chloriding' to be expected in solutions with chloride ions. This did not prevent the passage of adequate currents. On the basis of these findings silver wire and gauze were used for many of the tissue-holding electrodes; silver rather than gold, as suitable silver gauzes were available and silver has been much used for these purposes in electrophysiology. Lead-in wires were frequently of platinum electroplated with the catalytically inert gold. Gold was chosen for this rather than silver as gold was more resistant to the acids normally used in cleaning the vessels.

Tungsten and tantalum were also found suitable as electrode metals in that no gas changes occurred with the media and with the currents described above; also they could be sealed through glass.

Stainless steel could remain inert for long periods while in the polished condition in which it was purchased. Occasionally, however, patches of rusting commenced, sometimes associated with appreciable oxygen uptake. Iron, tin and lead were unsuitable as electrodes.

Choice of materials for supporting the tissue-holding electrodes. Except in the simplest case, electrodes were supported by fixing them in a plastic material. Specimens of these, both as pieces about the size of the intended holders and as chips, were shaken for some hours in ordinary manometric apparatus in (*a*) phosphate-saline and oxygen, and (*b*) bicarbonate-glucose-saline and oxygen-carbon dioxide mixture. Very small changes were observed with Polythene (a polyethylene) or Perspex (a methylmethacrylate resin), which were therefore chosen for holders. Bakelite preparations were less satisfactory.

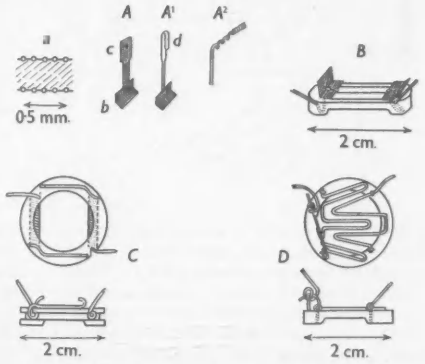

Fig. 2. Tissue-holding electrodes. *a*, diagrammatic drawing of gauze and slice (see text); *A*, hanging gauze electrodes; A^1, electrodes of gauze and wire (the flattened wire (*d*) can be replaced by the helix (A^2); A^2, helix for connecting electrodes A^1, *B*, *C* or *D* to lead-in wires; *B*, two electrodes of type *A* fixed to a Polythene holder; *C*, *D*, wire electrodes in Perspex holders.

Tissue-holding electrodes ('holders'). Type A. It was supposed that a suitable initial design for electrodes would be one which allowed electrical contact with both poles to an appreciable area of a tissue slice, while the tissue itself should have as great as possible a contact with a well aerated saline solution, i.e. should be entirely submerged in a mechanically agitated saline, as is normal in biochemical but not in electrophysiological experiments. The simplest arrangement then was to make electrical contact with the tissue by a metal gauze. Ag gauze is available, made from wires of 48 s.w.g., or 0·0406 mm. diameter, and 0·127 mm. apart. A scale drawing of part of a tissue slice 0·35 mm. thick, in such a gauze, is given in Fig. 2, *a*. It appeared that such a gauze would not greatly disturb diffusion to the slice. Warburg (1923) calculated that a tissue respiring at the rate of 0·05 ml. O_2/ml. tissue/min. would receive adequate oxygen from a solution saturated with the gas at 1 atmosphere, if it was less than 0·047 cm. thick. The rate of respiration of unstimulated cortex during our experiments has been 50–65 μmol./g./hr., or about 0·02–0·025 ml. O_2/ml. tissue/min. It increased during stimulation up to 0·04–0·05 ml./ml./min. We have, therefore, in common with other investigators, accepted Warburg's observations and used slices of about 0·035 cm. thick, which by the same means can be calculated to be fully oxygenated when respiring at the higher rate quoted above. Field's (1948) observations are consistent with this.

The gauze used for electrodes was cut to the shape shown in Fig. 2, *A*. Two such electrodes were used for each slice. One was taken, its flaps, *b*, were partly folded, and were submerged in a dish of saline containing a tissue slice. The gauze was held at *c*, the slice manoeuvred with a spatula (blade about 12 × 5 mm.) between the jaws of the gauze, and these gently closed on it with the spatula or with forceps. In doing this, pressure was applied only at the hinge of the flaps, so that the only pressure on the slice was that transmitted by the light gauze. The second electrode was then attached to the slice. The gauze had sufficient elasticity to maintain slight pressure on the slice for long periods after being put in place. (After many experiments the holder broke at the hinge and was renewed.) The ends *c* of the two electrodes were then brought together and picked up in forceps with the slice hanging below; excess saline was drained from the whole, and it was dropped to an experimental vessel with ends *c* uppermost. They were then hung on the lead-in wires and any saline removed from the junctions by filter paper.

Such electrodes are very flexible and light (about 20–30 mg. each) and with careful handling their attachment does not visibly harm the slice. A slice was commonly torn with electrodes of similar pattern, made from gauze of stainless steel, because of the greater strength and stiffness of this material. The slice can be removed from silver holders at the end of an experiment by opening their jaws with a spatula and floating away the slice on a dish of saline. During the shaking of a manometric experiment the electrodes and slice may swing freely, or occasionally one gauze electrode becomes attached to the side of the flask by capillary attraction. In a few instances when this has happened electrical contact with the lead-in wire has been lost. This, and the desirability of maintaining the electrodes in fixed positions in relation to each other, led to making the holders described below. After initial experiments the electrodes *A* were, however, also used in a few experiments to ensure that effects of added substances were not affected by absorption on plastic holders.

Holders type B. Modifications A^1 and A^2 (Fig. 2) were made in electrodes *A* with the intention of ensuring contact with the lead-in wires. The contact was to silver wire, flattened to a springy clip (A^1) or curled to a helix (A^2). In each case the silver wire was flattened at its lower end and threaded through and gripped to the gauze. These electrodes were, however, found to be too heavy and rigid for handling in the manner described above. A slice tended to be torn by relative movement between the pair. They were therefore fixed in insulating holders of different sorts, and of these the most suitable was *B*, Fig. 2. Such holders were made from polythene sheet, their shape being chosen to allow as free as possible a circulation of saline, and to allow the holder to be put in vessels such as *A* and *B* of Fig. 3. The parts of the wires not in contact with tissue or lead-in electrodes were insulated. In use, the holders were supported in a dish of saline with their jaws submerged, open to the extent indicated in Fig. 2, and carefully freed from air bubbles. Bubbles could completely prevent the entry of a slice. One edge of a tissue slice was then floated to one jaw, this closed as before, and the other end gripped by the other jaw. The holder was then held by one electrode wire and inserted, the other end first, into a vessel. One electrode after the other was then threaded on the lead-in wires of the vessel.

Holders type C. To allow a greater variety of electrode shapes and patterns to be made with greater precision, Perspex was used as the supporting material. Holders were made of basic patterns shown in *C* and *D*, Fig. 2; modifications of this are described below. In many of these, wire rather than gauze was used to obtain contact with the slice. This was maintained on the slices at a constant pressure by the springs shown. In one case this is a helix and in the other a safety pin or brooch-type of fastening is used. The wires held the slice against a grooved and scored portion of the holder, which ensured that the slice was fixed and in contact as much as possible with the saline. In use, the holder was immersed in a shallow dish of the saline used in the experiment and its jaws were held open by forceps which also held the whole holder firmly in position in the dish. A tissue slice was floated into position with a spatula, perhaps using also a camel-hair brush, and the jaws closed upon it as lightly as possible, and without any lateral movement which would crush and smear the tissue. Electrodes *C* required one end of the tissue to be put first in one electrode, and then the other end in the other.

Manometric vessels. In all cases lead-in wires ran from inside the vessel to a tube which could be filled with mercury (Fig. 3, *a*). Into this dipped a wire which carried the tongue of a push-in connexion. The wire and tongue were held in position by rubber tubing which also made the joint watertight. The tongue fitted into a socket leading to the source of current. This was often through a two-pole switch to a manifold which supplied several vessels (Fig. 1).

Vessels *A* (Fig. 3) are those already described. When the smaller vessels carried gauze electrodes *A* or *B*, 3–4 ml. of saline were adequate to cover the slices. Aerobic or anaerobic experiments were run with the usual gas mixtures in the vessels and absorbents in the centre well. Any additions made during the experiments were made from the side arm by tipping. The larger vessels A^1 required 7–10 ml. of saline for use with typical holders.

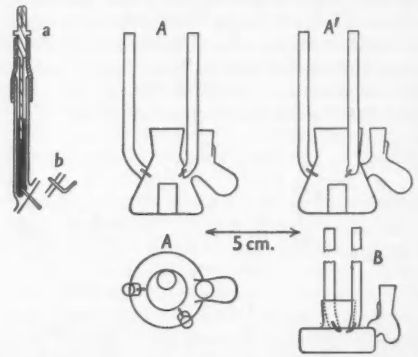

Fig. 3. Reaction vessels. *a*, arrangement of lead-in electrode suitable for tissue holding electrodes A^2, *B* and *C* (lead-in *b* suitable for tissue-holding electrodes *A*). Vessel *A*: conical, of about 20 ml. with side arm, two electrodes and eccentric centre well. Suitable for electrodes *A* and *B*, with approx. 50 mg. tissue and 3–4 ml. metabolic saline. Vessel A^1: as *A*, but of about 35 ml. and suitable for 100 mg. tissue and 6–10 ml. saline with electrodes *C* and *D*. Centre well again eccentric. Vessel *B*: cylindrical, of 10–17 ml.; with two electrodes and small side arm. For applying Warburg's indirect method for CO_2 and O_2 exchange. Used with electrodes *B* and 3–8 ml. of bicarbonate saline.

Vessels *B* were used for measuring respiration in bicarbonate-containing solutions by Warburg's (1924) improved method. They differ from Warburg's apparatus in several ways. For ease of manufacture they were made round in horizontal section, and not rectangular. Valve stoppers were fitted to small side arms from which 0·1 ml. of solution could be added. This addition was adequate for many purposes; provision of larger side arms would have made the results obtainable much less accurate. Lead-in wires were provided, by which electrodes *B* could be attached.

Vessels *C* (Fig. 4) were designed to admit a tissue-holding electrode as large as possible and to provide for gas-absorbing reagents, while keeping the total volume of the system as small as possible. Here the reaction was carried out in the large centre well and the annular space used for potassium hydroxide or

other reagents. With this design lead-in wires were provided either in the vessel or in a manometer such as that shown in Fig. 4.

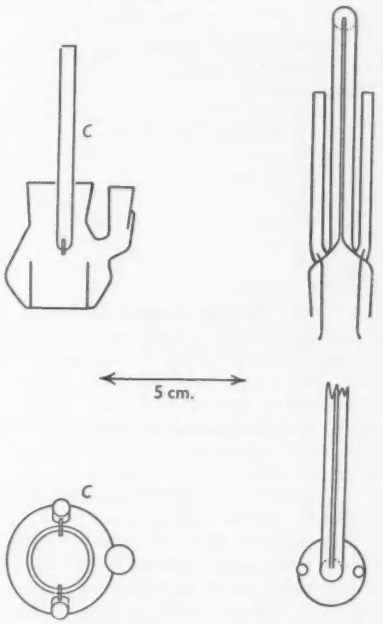

Fig. 4. Reaction vessels *C*; manometer with two electrodes. Vessel *C* designed to allow the use of electrodes *C* and *D* in a relatively small bulk of saline (approx. 3 ml.) in the centre chamber; absorbing reagents placed in the annular space.

Experimental procedure

The thermostat was brought to 37° and the source of electrical impulses tested. The salines were prepared and saturated with the appropriate gas mixture. The manometers to be used were greased on their cones, connected to a gas distributor and the gas flow adjusted. The reaction vessels were fitted with mercury and with the plug-in contacts; the valve stoppers were greased, any necessary addition made to the side arms and the stoppers inserted. The animal was killed and its brain sliced. As each slice was cut, a second worker trimmed the slices to size and weighed them. The first worker fitted slices to the electrodes. Salines were pipetted into the reaction vessels and the electrodes with their slices were fitted to the lead-in wires of the vessel. Any gas-absorbing reagents were added and the vessels attached to the manometers. Gas was passed for 5 min. with occasional manual shaking, the valve stoppers of the vessels closed, and the manometers and vessels put into the thermostat. After 3 min. the vessels and stoppers were worked firm and shaking commenced. Shaking was a reciprocating motion in a horizontal plane, operated by a crank and normally at the rate of 80 excursions of 8 cm. in each direction per min. It was interrupted to make the electrical connexions and to take readings of pressure of the manometers. The first reading was usually taken after 10 min. in the bath. This was 25–40 min. after the death of the animal.

When mains a.c. was used as the source of current, a suitable transformer setting was found to give the voltage required. The output of the transformer was then connected to the vessels. The voltage and current through each vessel was measured during the experiment. When condenser discharges were used, the charging voltage was selected and measured during the manometric experiment. The characteristics of the discharge in the particular vessels used were followed during the experiment with an oscillograph.

RESULTS

Respiration of slices in tissue-holding electrodes

Holding a tissue slice in a fixed position in a manometric flask would not be expected to affect its metabolism. In Warburg's (1923) early apparatus, slices were impaled on thin glass projections from the base of the vessels. Any damage to brain tissue, and any lack of contact with the saline in aerobic experiments, would be expected to lead to lowered respiration and increased anaerobic processes.

Table 1 contains the results of about forty experiments with the apparatus described above in each of which slices from a brain were incubated (i) free, and (ii) fixed in holders of various sorts. The average rate of respiration with fixed slices was slightly higher than that with free slices. In the case of the experiments of group 10, Table 1, the increase was statistically significant. This may possibly result from the fashion in which the slice is kept permanently extended in the holders, or from some effect of the wire itself. About half the experiments concerned outer slices of cortex, with one cut surface, and about half concerned inner slices with two cut surfaces. No difference was found between the two sets of experiments.

Effects of electric currents in the absence of tissue

Several of the experimental conditions involved passing appreciable currents through different physiological salines and metabolic mixtures. The greatest currents were from mains a.c. supplies, and the effect of these on the medium alone was accordingly examined. The supply was used at up to 3·5 V. with currents up to 100 ma./vessel, but usually not more than 20 ma. In control experiments such currents have been passed between gold, silver, and polished stainless steel electrodes in each

Table 1. *Respiration of brain-cortex slices in tissue-holding electrodes*

(Normally two equal electrodes held the slice at opposite ends; the area given is the sum of each at all their points of contact with the slice (i.e. above, as well as below). Areas given for gauze are those of the gauze, not of the wires which constituted it.)

Group no.	Tissue-holding electrodes: Metal	Area (sq.mm.)	Medium*	Respiratory rate in holder / Respiratory rate free × 100
	(i) Holders of wire (*w*) or gauze, hanging freely			
1	Pt	13	*a*	106, 115†, 100
2	Pt	65	*a*	110, 94, 106, 113†, 115, 96
3	Pt	400	*a*	91
4	Pt	65	*b*	110
5	Pt	65	*c*	101
6	Ag	60	*a*	120
7	Ag	60	*d*	116, 98, 100
8	Au	30 (*w*)	*d*	94
9	Stainless steel	65	*d*	92
	(ii) Holders of wire (*w*) or gauze in plastic supports			
10	Ag	80	*d*	110, 110, 103, 116, 105, 105, 89, 105, 109, 100, 104
11	Stainless steel	14 (*w*)	*d*	110, 87, 100, 100, 102
12	Stainless steel	14 (*w*)	*e*	96
13	Stainless steel	36 (*w*)	*d*	102
14	Stainless steel	36 (*w*)	*e*	105

* Media: *a*, glycylglycine, glucose, Ca^{++}; *b*, as *a*, without Ca^{++}; *c*, glycylglycine, glutamate, Ca^{++}; *d*, phosphate, glucose, Ca^{++}; *e*, phosphate, glutamate, Ca^{++}.

† With rat cortex; all others were with guinea pig.

experimental saline in oxygen or oxygen/5 % carbon dioxide without any progressive gas change. Cellophan and filter paper have been gripped between the electrodes in some experiments. Thus neither electrolysis, oxidation, nor formation of acidic and basic materials has been caused. Untoward effects with platinum electrodes are noted above.

Gas changes due to the heating effect of the currents have, however, been observed. A typical effect is shown in Fig. 5. Passage of the current caused a small increase in pressure, which within about 2 min. was stabilized at a slightly higher value than before. It sank to its original value on switching off the current. Presumably, therefore, the vessel is maintained by the current at a temperature slightly above that of the thermostat, but no progressive increase in temperature takes place as heat is removed by the water of the thermostat. In different experiments the increase in pressure has been found to be greater with the passage of greater currents. By applying the gas laws, the mean increases in temperature in the vessels were calculated to be of the order of 0·03–0·3° in typical experiments (see Table 2). The calculation was made by simple proportionality between pressure (1 atm. = 10,000 mm. of manometric fluid) and temperature (originally 310° K.), and solubility differences were not taken into account. The validity of this calculation can be confirmed, at least approximately, by calculation on an altogether different basis. The electrical energy expended in the vessels can be obtained from the characteristics of the currents used, and the temperature gradient needed for its dissipation calcu-

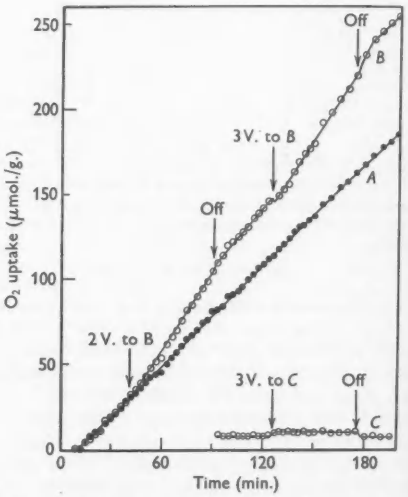

Fig. 5. Respiration of (*A*) a free, outer, guinea pig brain-cortex slice and (*B*) a similar slice, stimulated. The stimulated slice was in a holder of type *B*, Fig. 2, in a vessel of type *A*, Fig. 3. Gas phase: O_2, with NaOH paper in the centre well. Medium: glucose phosphate saline with Ca. Stimulation: 50 cyc./sec. a.c. at 2 and 3 V. as indicated. Curve *C* represents the pressure change in a vessel with medium and electrode only, calculated as though the vessel contained a slice of the same weight as the experimental slices. The current causes an *increase* in pressure, as shown in *C*; this causes a momentary *decrease* in apparent O_2 uptake, as shown in *B*.

Table 2. *Heating effect of currents during manometric experiments*

Vessel type	Electrodes	Potential applied (V.)	Current passing (ma.)	Pressure increase (mm. of manometric fluid)	Calculated mean temperature increase (°)
		Alternating current			
A	*B*	3·0	8	3	0·10
A	*C*	3·0	23	6	0·20
		3·0	48	11	0·34
B	*B*	1·9	13	2	0·06
		2·9	20	3	0·09
B	*B*	1·5	11	1·5	0·05
		2·2	17	2	0·06
		Condenser discharge			
A^1	*C*	6	(100/sec., 4 μF.)	<1	<0·03
A^1	*C*	24	(100/sec., 4 μF.)	4	0·12

lated from the thermal conductivity of glass and dimensions of the vessels. The energy put to the cells by 30 ma. at 3 V. corresponds to about 0·02 cal./sec.; that by 100 pulses/sec. from a 4 μF. condenser at 24 V., to about 0·03 cal./sec. In glass with conductivity of 0·002 cal./sec./cm. thickness/sq.cm./°, as a vessel of 60 sq.cm. area and 1 mm. thickness, loss of 0·03 cal./sec. would require a temperature difference of about 0·03°.

The mean temperature change is thus small enough to be ignored. If the temperature change were highly localized within the vessel it could still be serious, but there is no indication that such localization occurs. The current, and the heat generated by it, would be expected to be spread through a considerable proportion of the saline medium. That this is the case was shown by altering the position of the electrodes. When they were at the surface of the liquid, the current which passed between them was approximately half that which passed when they were fully submerged. This occurred whether or not the slice was gripped in the electrodes. The slice had a much lower conductivity than the medium. The effect of temperature on the respiration of slices of cerebral cortex from the rat has been studied by Field, Fuhrman & Martin (1944) and Fig. 2 of their paper shows about 30 % increase in rate between 37·5 and 42·5°. The higher rates also are not well maintained. It appears unlikely that temperature changes would cause major artifacts in the present experiments.

The increase in pressure due to heating can also be recognized as superimposed upon progressive changes of pressure due to reactions occurring simultaneously in the manometric vessels. An example in which the progressive change is caused by respiration of a brain slice not attached to the electrodes is given in Fig. 7. The pressure change due to heating can then be obtained by extrapolation, and values so obtained are close to those of Table 2. The same heating occurs when a slice is held between the electrodes (Figs. 5–8). It can be clearly differentiated from the other changes occurring under these conditions by its magnitude, and by the fact that it occurs promptly and is reversed promptly on switching off the current.

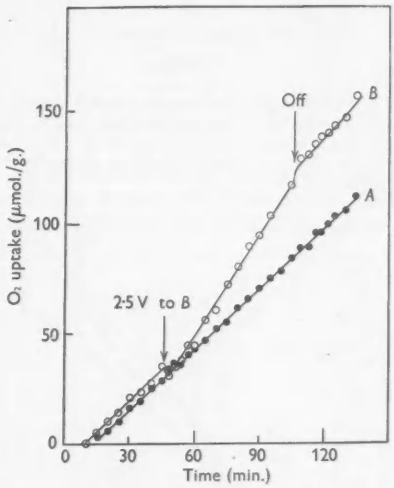

Fig. 6. Respiration of unstimulated (*A*) and stimulated (*B*) inner slices, both in holders of type *D* in vessels A^1. Stimulation at 2·5 V.; other details as Fig. 5.

Respiratory response to applying potentials to nervous tissues

Increased oxygen uptake has been found to follow the application of varying potential gradients in a variety of experimental circumstances. The increase is very easily observed in the apparatus described, but one of our main concerns has been to investigate whether or not it represents a genuine response of the tissue to the applied potentials. Experiments

have therefore been arranged in a number of ways so that alternative explanations could be examined.

(1) *Comparison with control slices.* Slices in normal Warburg vessels have been included in most experiments as a criterion of the normal behaviour of

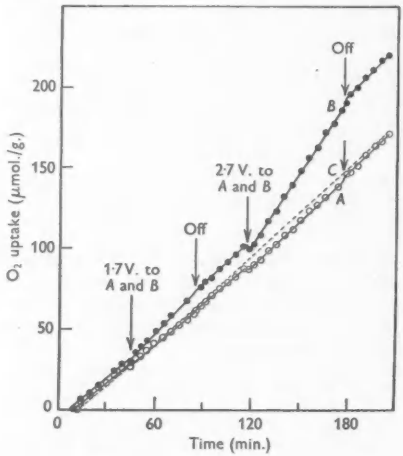

Fig. 7. Respiration of (*A*) a free outer slice in a vessel type A^1, Fig. 3, also containing an electrode type *C*, Fig. 2; (*B*), a similar slice held in the electrode in such a vessel; (*C*) a free slice in a vessel without an electrode. Other details as Fig. 5.

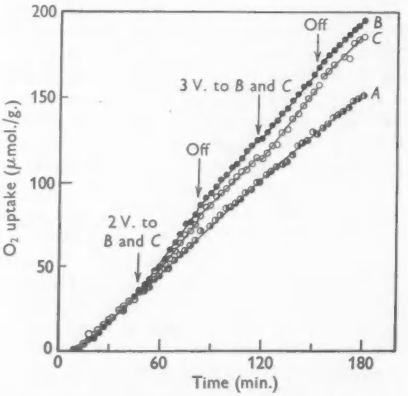

Fig. 8. Respiration of slices: (*A*) free; (*B*) in holders *B* (Fig. 2) and vessels (*A*) (Fig. 3); (*C*) as (*B*) but cut halfway between the two electrodes.

medium and slice. The difference during stimulation is clear (Fig. 5). Slices in holders, but not stimulated, have been compared with those which are stimulated (Fig. 6), and again show a clear effect of stimulation.

(2) *Comparison with the same slice in absence of stimulation.* Most experiments included periods with and without stimulation; the effect of the applied current is then as shown in Figs. 5–8.

(3) *Comparison with similar vessels not containing slices.* If the increase in gas absorption is due to the current alone, a progressive increase should be seen in an otherwise empty vessel in the absence of tissue. Fig. 5, however, shows that the only phenomenon under these conditions is the transitory increase in pressure presumably (see above) caused by heating. Any chance difference between vessels and electrode arrangements was eliminated by altering the arrangements so that a slice was stimulated in the vessel previously used as a control.

(4) *Comparison with similar vessels containing electrodes and slices not attached to electrodes.* This comparison was made because the slices change the composition of the medium and might conceivably produce substances labile at the applied potential. No indication of this was found. Vessels with slices attached to electrodes showed increased oxygen uptake; others did not. Fig. 7 illustrates this.

(5) *Various other phenomena* described below can also be regarded as evidence that changes observed are a response of the tissue.

Dependence of response on slice/electrode arrangements

As is indicated by the results of paragraph (4) above, a fairly close relationship between slice and electrode is needed to obtain maximum respiratory

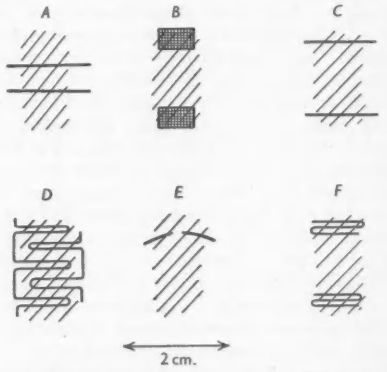

Fig. 9. Electrode patterns (see text). *B* is the same as *B* of Fig. 2; all others were of wire fitted to Perspex holders *C* or *D* of Fig. 2. The approximate position of a tissue slice is indicated by shading.

response with a given electrode and stimulus. To obtain this, light pressure has been exerted by the electrodes on the tissue. Various arrangements of electrodes and tissue are possible of which we have examined several illustrated in Fig. 9. Table 3 shows

Table 3. *Increase in respiration caused by electrodes of different types*

(Exps. 1–9 were made with guinea pig cerebral cortex in glucose phosphate saline with Ca salts, and an atmosphere of O_2 in vessels *A* (Fig. 3) or *C* (Fig. 4). Exp. 10 was in glucose bicarbonate saline with Ca salts, and CO_2/O_2, in vessels *B*. Stimulation was by mains a.c. through a transformer at the voltage quoted except in Exp. 9. This employed discharge from a 4 μF. condenser of about 1 msec. duration, at the peak voltage quoted. The first period of stimulation was normally about 40 min. and the second 120–140 min. after putting the vessels into the thermostat. Respiration is calculated as 100 × rate during stimulation/mean of rate by the same slice in periods immediately before and after stimulation. The mean value before and after stimulation was 57 mol./g. wet wt./hr. corresponding to 9 μl./mg. dry wt./hr.)

Exp. no.	Electrode type (see Fig. 9)	Slice (*o*, outer *i*, inner)	Stimulating period	Voltage (V.)	Current (ma.)	Respiration (% of control)
1	*E*	*o*	1st	1·6	8	109
1		*o*	2nd	2·5	19	120
2	*E*	*i*	1st	2	23	105
2		*i*	2nd	3	36	107
3	*A*	*i*	1st	1·6	7	116
3		*i*	2nd	2·5	17	141
4	*C*	*o*	1st	2	28	138
4		*o*	2nd	3	41	160
5		*o*	1st	2·5	28	174
6		*i*	1st	1·6	10	118
6		*i*	2nd	2·5	24	136
7	*F*	*i*	1st	2·3	16	157
7		*i*	2nd	2·3	16	147
8	*D*	*i*	1st	2·1	90–100	208
8		*i*	2nd	2·9	120–130	200
9	*D*	*o*	1st	6	See above	180
9		*o*	2nd	12	See above	200
10	*B*	*i*	1st	2·9	13, 17	179

that electrodes (*E*), which give a relatively small area of contact with the slice and offer a fall of potential of 2–3 V. over a small space of about 2 mm., caused little stimulation. When the current passed between two parallel wires with much more of the slice between them, as in electrodes *A* and *C*, larger responses were given. The currents passing were comparable. There was not a great difference between the responses given to arrangements *A* and *C*, although a much greater proportion of the slice is between the electrodes in *C*. Increasing the area of each electrode, as in *F*, while still leaving a large proportion of the slice some mm. from each electrode, did not greatly increase the response. Greater response was, however, obtained with the grid *D*, in which each part of the slice is within 1–2 mm. of the electrode and the potential gradient is higher.

Dependence on time and length of stimulation

Long or repeated stimulation led to falling response by the tissue. The period during which a response was obtained, however, extended for some hours. An example is quoted in Table 3 in which response to a second period of stimulation at a given level was less than to the first. Also, response to a given voltage was less when this was applied late in an experiment than when it was applied early (see Fig. 10). Maintenance of response did not markedly differ when condenser discharges were compared with the sine-wave alternating current.

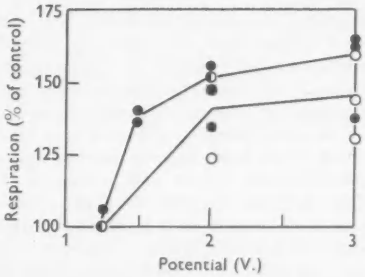

Fig. 10. Respiration of guinea pig brain slices in holders *B*, Fig. 2, at varying voltages. Glucose phosphate saline in O_2. Black points and upper line: first period of stimulation beginning after about 40 min. metabolism. Open circles and lower line: second period of stimulation beginning after about 120 min. Lines drawn through mean values.

Dependence on applied potential

As is indicated above electrical energy can be expended in a vessel containing tissue without having any effect on the metabolism of the tissue.

When the tissue was fixed in close proximity to electrodes, the metabolic response varied with the voltage applied (see, for example, Fig. 7). In the electrodes examined, a threshold voltage has been found for increase in respiration; this was of the order of 1·5 V. for sine-wave a.c. (Fig. 10). Above this the response increased rapidly and then more slowly; the maximum increase observed was of the order of 100 % (Table 3).

DISCUSSION

In judging the significance of the effects reported above, it is important to compare them with existing knowledge of the response of the brain to electrical stimulation *in vivo*. Many similarities are found. Because of this it appears reasonable to refer to the applied potentials in the present experiments as 'stimuli'. The features which can be compared are as follows.

Electrical characteristics. Full examination will be reported later. In most of the present experiments the type and frequency of the stimulus has been chosen arbitrarily. However, when that choice has been made, it is significant that the voltage needed for metabolic response *in vitro* is not far from that found necessary for autonomic responses *in vivo* by Bailey & Sweet (1940); for motor responses *in vivo* by Clark (1939), Moruzzi & Magoun (1949) and Wyss & Alcade (1937), or for the electrical response *in vitro* by Libet & Gerard (1939). The comparison cannot, however, be pressed as the electrode arrangements differed among the experiments *in vivo* and differed from our own.

Promptness of response. Curves such as those of Figs. 5–8 suggest that the metabolic response is maximal within 1 or 2 min. of the commencement of stimulation. It is thus as prompt as can be detected by the present apparatus. Motor responses *in vivo* occur within fractions of a second and increased respiration of the brain *in vivo*, measured potentiometrically, occurs within half a minute (Davis, McCulloch & Roseman, 1943; Gurdjian, Webster & Stone, 1946). The findings *in vivo* and *in vitro* therefore agree within the limits of measurements at present available. Increased metabolism in the present experiments usually ceased fairly promptly on stopping the current In some cases an increased rate may have persisted for a few minutes after stimulation.

Fatigue. Appreciable falling in response occurs only after some hours; i.e. after passing perhaps 10^6 impulses. The tissue *in vitro*, as *in vivo*, is thus very resistant to fatigue.

Respiration. Respiration increases on applying adequate potentials both *in vivo* and *in vitro*. The magnitude of the response observed *in vitro* is within the range of metabolic potentialities recorded for the brain *in vivo* and *in vitro*. Thus during convulsions the rate of respiration of brain as measured by arterial/venous difference can increase to 180 % of its normal values (Schmidt *et al.* 1945). The unstimulated rates of respiration of cortex slices in our experiments are similar to those of many others. They correspond to values of about 10 μl./mg. dry wt./hr. Such rates are, however, known to be increased by glutamate (Weil-Malherbe, 1936) or by absence of calcium salts (Dickens & Greville, 1935; Canzanelli, Rogers & Rapport, 1941). By these and other modifications in physiological salines, rates of respiration of brain cortex *in vitro* have recently been observed (Krebs, 1950) which are two to three times those of ordinary unstimulated slices. The increases which we observed on electrical stimulation are therefore within the ordinary metabolic potentialities of the tissue, and can be induced by other means. Whether these other means alter an electrical state in the slices is at present unknown.

The present results can also be compared with the effects of stimuli on other separated systems. Observations with peripheral nerve are most closely analogous, and show that increased respiration also follows electrical stimulation. A new feature of the present work lies in the demonstration of such relationships after the much greater disturbance involved in slicing a highly complex organ, and the application of neurophysiological techniques to tissue slices under optimal biochemical conditions. Here it may be noted that the respiration of tissue slices from the submaxillary gland was found by Deutsch & Raper (1938) to be increased by acetylcholine.

The means by which electrical stimulation of the brain *in vivo* leads to increased respiration is through the induction of increased electrical activity by the tissue itself. The present findings suggest that cortex slices may be capable of showing induced electrical activity and that this may represent a modification of electrical conditions pre-existing in the slice. The rate of respiration of brain-cortex slices in ordinary physiological salines is closely similar to the rate found for the normal (electrically moderately active) organ *in vivo* (Schmidt *et al.* 1945; Elliott & Henderson, 1948). It has already been noted that Libet & Gerard (1939) found small isolated fragments of certain parts of frog brain to exhibit spontaneous potential changes *in vitro*, and found such changes to increase with applied stimuli. Respiration was not then measured. de Barenne & McCulloch (1941) and Burns (1950) have found electrical responses to stimulation in portions of cerebral cortex still supplied by the bloodstream of the animal, but otherwise separated; the cortex did not show spontaneous activity.

These investigations, together with our own, emphasize how autonomous are separated portions

of brain tissue. They suggest that separated portions of cerebral cortex from experimental animals or from man may be used in a wide range of investigations in which metabolic and electrical responses to stimuli are open to simultaneous investigation.

SUMMARY

1. Methods have been devised, using modified manometric apparatus, which enable electric stimuli to be applied to thin slices of brain tissue under good metabolic conditions.

2. The application for some hours of alternating currents of up to 3 V., or of brief condenser discharges of up to 30 V. several time per sec., between electrodes 2–10 mm. apart caused in the apparatus no changes which might be mistaken for metabolic responses.

3. When current was applied to electrodes in contact with brain-cortex slices, the respiration of the slices increased. Values up to double those of the initial respiration were observed.

4. The increased respiration could be maintained for some hours, and in a given experimental arrangement increased with increase in the voltage applied, when this was above a threshold value.

I am greatly indebted to Dr G. Anguiano, Mr J. D. Cheshire, and Miss S. Russell for assistance during these investigations; to Mr H. L. Buddle and Mr F. Ibbott for making apparatus; to Mr P. St John-Loe for electrical matters and to A. L. Hodgkin, Esq., F.R.S., for his helpful comments on the manuscript.

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Amino-acids and Proteins in Haemoglobin Formation

2. ISOLEUCINE

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Among essential amino-acids, the role of isoleucine in haematopoiesis is specially interesting, because, despite its absence in ox, human and horse globin (Brand & Grantham, 1946), this amino-acid has been shown to be essential for haemoglobin formation in weanling rats by Orten and collaborators (Orten, Orten & Bourque, 1945; Orten, Bourque & Orten, 1945). The present paper describes studies on the influence of isoleucine on the regeneration of haemoglobin and red cells in adult rats suffering from experimental anaemia, graded doses of isoleucine being administered to rats maintained on a diet containing horse globin as the sole source of protein and rendered anaemic by administration of

Table 1. *Recovery of rats from phenylhydrazine anaemia on a basal isoleucine-free diet and on diets containing different amounts of isoleucine*

(Red cell values in millions/cu.mm.; haemoglobin values in g./100 ml. blood; weight in g. Group means with their standard error.)

Exp. no.	No. of animals used	Isoleucine dosage (mg./rat/day)		Time after injection (days) 0	4	8	12	14	16	18	21	26	33
1	16	0	Red cell no.	6·37±0·19	3·66±0·26	4·41±0·58	5·32±0·48	—	5·93±0·79	6·19±0·38	6·41±0·44	5·72±0·54	4·66±0·79
			Haemoglobin	14·6±0·07	9·5±0·10	10·9±0·11	12·3±0·09	—	12·9±0·09	13·5±0·10	14·3±0·06	13·5±0·07	11·2±0·06
			Weight	134 ±7·8	121 ±7·5	113 ±7·1	—	107±6·5	—	—	101 ±6·2	91±5·2	83±5·1
2	8	10	Red cell no.	6·35±0·03	3·61±0·11	4·54±0·11	5·58±0·09	—	6·00±0·08	6·25±0·09	6·36±0·08	6·28±0·06	6·16±0·07
			Haemoglobin	14·6±0·12	9·5±0·19	11·1±0·23	12·6±0·16	—	13·2±0·16	14·4±0·13	14·6±0·09	14·5±0·12	14·1±0·10
			Weight	130 ±12·2	118 ±10·9	112 ±10·7	—	106±10·2	—	—	102±10·0	—	—
3	8	20	Red cell no.	6·39±0·04	3·47±0·09	4·38±0·06	6·37±0·05	—	6·43±0·05	6·44±0·05	—	—	—
			Haemoglobin	14·6±0·06	9·4±0·08	12·4±0·06	14·6±0·07	—	14·8±0·08	14·8±0·08	—	—	—
			Weight	136 ±7·3	125 ±6·8	119 ±6·9	—	115±5·7	111 ±6·1	—	—	—	—
4	10	20 (by injection)	Red cell no.	6·44±0·08	3·75±0·08	4·54±0·09	6·45±0·08	—	6·74±0·08	—	—	—	—
			Haemoglobin	14·2±0·08	9·8±0·16	11·7±0·19	14·1±0·07	—	14·4±0·06	—	—	—	—
			Weight	126 ±11·1	113 ±9·4	106 ±9·0	—	101±8·8	98±8·7	—	—	—	—
5	8	40	Red cell no.	6·43±0·04	3·42±0·05	4·38±0·05	6·43±0·04	—	6·47±0·04	6·50±0·03	—	—	—
			Haemoglobin	14·7±0·17	9·3±0·10	12·4±0·19	14·6±0·16	—	14·8±0·13	14·8±0·11	—	—	—
			Weight	139 ±9·2	126 ±8·7	122 ±8·4	—	119 ±8·1	116 ±7·8	—	—	—	—
6	8	100	Red cell no.	6·63±0·08	3·67±0·06	4·56±0·07	6·55±0·09	—	6·68±0·12	6·65±0·13	—	—	—
			Haemoglobin	14·4±0·08	9·4±0·11	11·9±0·15	14·4±0·07	—	14·5±0·09	14·7±0·07	—	—	—
			Weight	130 ±11·1	116 ±10·1	126 ±10·8	—	132 ±10·8	134 ±10·9	—	—	—	—

phenylhydrazine according to the technique described (Yeshoda & Damodaran, 1947). The effect of prolonged isoleucine deficiency on haematopoiesis in normal adult animals was also studied.

EXPERIMENTAL

Preparation of horse globin. Globin was prepared by a combination of the methods of Schulz (1898) and Anson & Mirsky (1930); it was found that when the latter method was used exclusively, very large volumes of acetone were required to give a satisfactory product. Horse red corpuscles, freed from serum by centrifuging of defibrinated blood and repeated washing with 0·9% (w/v) NaCl, were laked by the addition of ether and, after centrifuging to remove the stroma, dialysed free from NaCl. The solution was now treated at 0° with ice-cold 0·1 N-HCl in quantity just sufficient to redissolve the flocculent precipitate formed, the amount of acid required being determined by preliminary titration of a measured fraction. Half the volume of ethanol was added and then the pH brought carefully to 7·5 by the dropwise addition of NH_3. The precipitate formed was separated by decantation and centrifuging, washed free from haemin with acidified acetone (1 vol. of HCl/100 vol. of acetone), filtered at the pump and dried by washing with acetone and ether. The globin so prepared contained 0·4% ash and 16·85% N (moisture-free).

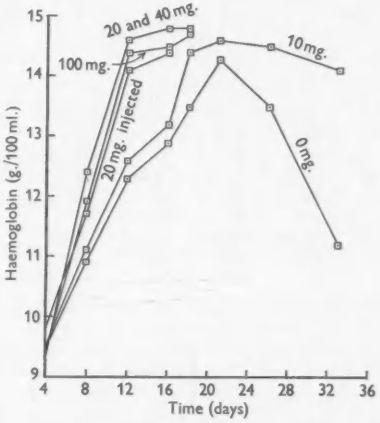

Fig. 1. Regeneration of haemoglobin after phenylhydrazine administration at varying daily levels of isoleucine. All the figures are mean values (for details see Table 1).

Preparation of isoleucine. L-Isoleucine was first prepared from casein phosphopeptone available from previous work (Damodaran & Ramachandran, 1941) by the method then described. Later it was prepared directly from casein. Casein was hydrolysed in the usual way with H_2SO_4, and after removal of acid with $Ba(OH)_2$, the solution was evaporated till tyrosine separated out. The filtrate was further concentrated to give a crystalline mixture of leucines. Isoleucine was isolated via the methanol-soluble Cu salt (Damodaran, 1931).

Experimental diets and animals. Experiments were carried out on rats from the stock colony weighing from 100 to 175 g., animals being as far as possible evenly distributed in groups regarding age, weight and sex. The isoleucine-free basal diet was made up as follows: horse globin, 18; tapioca starch, 40; sugar, 20; melted butter fat, 17; salt mixture, 5%.

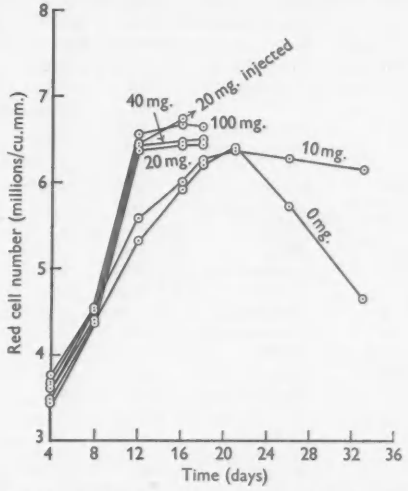

Fig. 2. Regeneration of erythrocytes after phenylhydrazine administration at varying daily levels of isoleucine. All the figures are mean values (for details see Table 1).

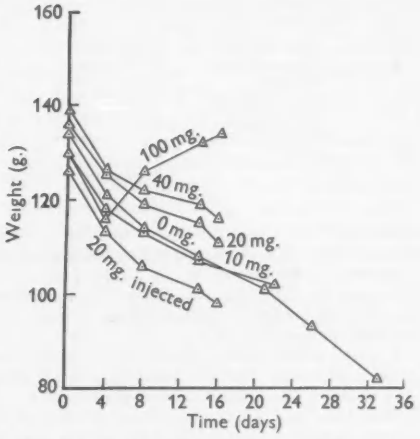

Fig. 3. Body weights of rats recovering from phenylhydrazine treatment at different daily levels of isoleucine. All the figures are mean values (for details see Table 1).

The latter was the Steenbock-Nelson salt mixture no. 40 +0·03% $CuSO_4$, $5H_2O$ (Steenbock & Nelson, 1923; Pearson, Elvehjem & Hart, 1937). Each rat received in addition

Table 2. *Effect of prolonged isoleucine deficiency in normal animals*

(Red cell values in millions/cu.mm.; haemoglobin in g./100 ml. blood; body weight in g.)

		Time (days)								
Rat no.		0	15	30	40*	45	50	55†	70	80
59	Red cell no.	7·37	6·27	4·71	4·02	5·22	6·41	7·08	5·41	4·08
	Haemoglobin	15·3	13·5	10·3	9·3	10·8	12·2	14·5	13·2	9·9
	Weight	184	149	130	114	—	110	94	71	53
60	Red cell no.	7·16	6·05	4·58	3·74	4·88	6·38	6·99	5·47	4·21
	Haemoglobin	15·2	13·5	10·4	9·3	10·6	12·1	14·6	13·3	10·1
	Weight	176	136	119	100	—	88	83	68	50
61	Red cell no.	6·58	5·37	4·42	3·81	4·83	6·44	7·11	Died	—
	Haemoglobin	14·6	13·2	10·1	8·9	10·6	13·1	15·2		
	Weight	109	72	68	59	—	51	48		
62	Red cell no.	6·66	5·41	3·63	3·14	4·46	6·57	6·97	5·53	Died
	Haemoglobin	14·4	13·4	10·5	9·5	10·4	12·8	14·7	13·1	
	Weight	128	93	70	61	—	57	52	41	
63	Red cell no.	6·82	5·58	4·11	3·35	4·67	6·84	7·14	5·68	Died
	Haemoglobin	14·8	13·2	10·2	9·1	10·5	12·8	14·6	13·7	
	Weight	110	81	70	53	—	48	42	38	
64	Red cell no.	6·30	5·17	3·42	Died	—	—	—	—	—
	Haemoglobin	14·3	13·0	9·8						
	Weight	104	88	63						
65	Red cell no.	6·28	5·04	3·39	Died	—	—	—	—	—
	Haemoglobin	14·4	13·1	8·6						
	Weight	104	68	51						
66	Red cell no.	6·49	5·39	4·61	3·87	5·17	6·57	6·81	5·58	Died
	Haemoglobin	14·5	13·3	10·4	9·5	10·7	12·9	14·2	13·1	
	Weight	106	88	69	51	—	46	42	39	
67	Red cell no.	7·08	5·83	4·17	3·44	4·82	6·48	6·96	5·51	Died
	Haemoglobin	14·8	13·2	10·3	9·3	10·6	12·7	14·5	13·3	
	Weight	138	100	81	64	—	48	44	39	
68	Red cell no.	6·71	5·64	4·08	3·51	4·78	6·29	6·92	5·62	Died
	Haemoglobin	14·6	13·2	9·9	9·1	10·6	12·8	14·4	13·1	
	Weight	117	81	52	64	—	58	53	44	
69	Red cell no.	6·56	5·24	3·89	Died	—	—	—	—	—
	Haemoglobin	14·2	13·4	9·9						
	Weight	104	65	52						
70	Red cell no.	6·37	5·21	3·76	Died	—	—	—	—	—
	Haemoglobin	14·3	13·2	9·3						
	Weight	103	60	43						

* Isoleucine supplemented. † Isoleucine withheld.

a standard cod liver oil concentrate corresponding to 180 i.u. of vitamin A and 30 i.u. of vitamin D/day, and the following supplements of vitamins of the B complex/day: 25 μg. aneurin hydrochloride, 20 μg. riboflavin, 20 μg. pyridoxin, 100 μg. calcium pantothenate and 100 μg. nicotinic acid. The animals on the isoleucine diet received daily in addition to the above, isoleucine at different levels administered separately. The food was provided *ad lib.* and the daily intakes recorded. The animals were weighed twice a week. The techniques used for the production of anaemia and estimation of red cells and haemoglobin were as described previously (Yeshoda & Damodaran, 1947).

Experiments on anaemic animals. Six groups of animals were used. The control group consisting of sixteen animals was maintained on the isoleucine-free basal diet. Four groups, each of which contained eight animals, received in addition to the basal diet 10, 20, 40 and 100 mg., respectively, of L-isoleucine/rat/day administered orally. To the sixth group 20 mg. of the amino-acid were given by subcutaneous injection. Initial red cell and haemoglobin values were determined in blood from the tail vein on 3 consecutive days preceding experiment. The animals were placed on the basal diet on the day on which phenylhydrazine was injected; isoleucine supplements were given from the 4th day when the peak of anaemia was reached. Determination of erythrocytes and haemoglobin were made every 4th day till the animals recovered from anaemia or till it became evident that there was no possibility of recovery. The mean values for haemoglobin, red cells and body weights for the six groups of animals are given in Table 1 and represented graphically in Figs. 1, 2 and 3.

Experiments on normal animals. A group of twelve animals was kept on the isoleucine-free basal diet and observations on red cells, haemoglobin and weights made for a period of 70–80 days. The results are recorded in Table 2 and Fig. 4.

RESULTS AND DISCUSSION

It will be seen from Table 1 and Figs. 1 and 2 that the course of recovery of animals from anaemia is clearly dependent upon the presence as well as upon the quantity of isoleucine in the diet. The animals on the isoleucine-deficient diet recovered their normal haemoglobin and red cell values on the 21st day of the experiment, or 17 days after the most acute stage of anaemia. The same was true of the animals receiving 10 mg. of isoleucine per day. On 20 mg. or more of isoleucine per day complete recovery took place within 8 days. Injected isoleucine also brought about recovery in the same length of time.

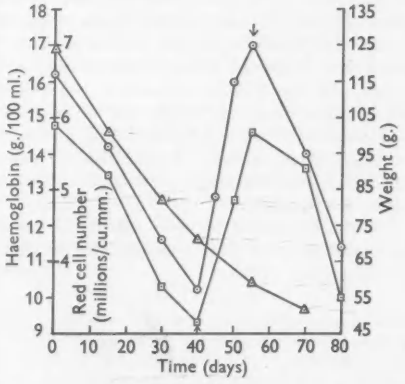

Fig. 4. Anaemia and loss of weight in prolonged isoleucine deficiency. ↑, isoleucine supplemented; ↓, isoleucine withdrawn. —⊙—, red cell number; —△—, body weight; —⊡—, haemoglobin constant. All the figures are mean values (for details see Table 2).

For statistical analysis the group averages of the changes in red cell number and haemoglobin content in the first 8 days from the peak of anaemia (4th–12th day), calculated as percentages of the initial values, given in Table 3 were used. 'Student's' test was applied for the calculation of 't'. Similar calculations were made for the percentage changes in weights also, but for a period of 10 days from the peak of the anaemia.

The values of 't' given in Table 4 leave no doubt as to the reliability of the interferences stated above. The analysis shows in addition that there is no significant difference in the rate of regeneration of erythrocytes or of haemoglobin at isoleucine intakes above 20 mg. As between injection and oral administration statistical analysis shows that the latter is significantly more effective for haemoglobin formation, although the overall time of recovery was about the same in both the cases.

Table 3. *Change in haemoglobin, red cells and body weight expressed as percentages of initial values*

Exp. no.	Dosage used (mg.)	Red cells 4–12th day	Haemoglobin 4–12th day	Weight 4–14th day
1	0	26·1	18·9	−11·8
2	10	32·4	21·3	− 9·2
3	20	45·3	37·1	− 7·5
4	20 (injected)	41·8	30·6	−10·2
5	40	46·7	36·4	− 5·1
6	100	43·3	35·2	+12·4

The amount of isoleucine administered has a pronounced effect on the weights of the animals, in marked contrast to the results previously reported on tryptophan. With the latter it was found that 20 mg. of the amino-acid per day, which was required for normal haematopoiesis, was also sufficient to prevent loss of weight of the animals recovering from phenylhydrazine anaemia, except when the tryptophan was given by injection. A difference of the same kind between oral and parenteral administration was observed in the case of isoleucine too, but even under conditions of oral administration loss of weight was prevented only at a level of 100 mg. of isoleucine, although a fifth of this quantity was found sufficient for normal haematopoiesis. These observations are in accord with the results of Frost & Sandy (1948) who, in assays on the optimum level of tryptophan and isoleucine in partial hydrolysates of fibrin, found the requirement of tryptophan for maximum gain in weight to be 18–20 mg. per day and of isoleucine somewhat greater than 75 mg.

In another important respect also the effects observed in isoleucine deficiency differ from those

Table 4. *Statistical analysis of percentage changes in haemoglobin, red cells and body weight*

(Values of 't'.)

		Haemoglobin					Red cells					Weight				
Isoleucine dosage	...	10	20	20 (injected)	40	100	100	40	20 (injected)	20	10	100	40	20 (injected)	20	10
Dosage	0	1·66	10·43	8·17	12·73	10·58	14·45	23·51	12·14	16·13	3·58	14·65	4·27	1·08	2·71	1·61
compared	10	—	9·03	7·27	19·16	10·62	4·16	6·12	3·69	4·93	—	21·28	6·18	1·09	2·31	—
with	20	—	—	3·63	0·43	0·98	1·09	0·99	1·81	—	—	21·92	4·99	3·30	—	—
	40	—	—	—	—	1·06	2·43	—	—	—	—	20·80	—	—	—	—

reported with regard to tryptophan. Although the recovery of animals from anaemia was greatly retarded by the absence of tryptophan, the blood picture was ultimately restored to normal, presumably at the expense of body substance as the animals showed great loss in weight. In agreement with this result attempts to make normal animals anaemic by removal of tryptophan from the diet were unsuccessful. Rats maintained on a tryptophan-deficient diet lost weight and died in 58–75 days, but throughout the experimental period exhibited no anaemia, the haemoglobin content and erythrocyte count showing no statistically significant difference from animals on a complete diet. In isoleucine deficiency, on the other hand, the recovery from phenylhydrazine anaemia was not only delayed, but was also temporary (Table 1 and Figs. 1 and 2). The blood picture was restored to normal on the 21st day of the experiment, but marked anaemia set in about the 33rd day. These results were fully confirmed by experiments on normal animals maintained on globin for an extended period. The results of this experiment which are detailed in Table 2 and Fig. 4 show that the blood picture rapidly responds to the presence and absence of isoleucine. Noticeable fall in haemoglobin and red cell values took place in a fortnight on an isoleucine-deficient diet, and in the course of about 40 days acute anaemia comparable to that produced with phenylhydrazine set in. On addition of 20 mg. of the amino-acid the blood picture returned to normal in 15 days. By the withdrawal of isoleucine from the diet anaemia could be again induced in these animals which died at the end of 70–80 days in the second period of isoleucine deficiency.

SUMMARY

1. The effect of administering isoleucine at various levels was studied on growth, red cell number and haemoglobin content of animals made anaemic by phenylhydrazine injection and maintained on a basal diet containing no isoleucine. The effect of prolonged isoleucine deficiency on normal animals was also investigated.

2. It was found that isoleucine is essential for haematopoiesis in rats, regeneration of erythrocytes and haemoglobin in animals suffering from experimental anaemia being greatly retarded in the absence of this amino-acid.

3. The quantity of isoleucine required for maintenance of weight was much greater than that required for haematopoiesis. Administration of quantities of isoleucine greater than 20 mg. per rat per day did not further accelerate red cell and haemoglobin formation, whilst for prevention of loss in weight about 100 mg. was found necessary.

4. Injected isoleucine was utilized for haemopoiesis, but was less effective than isoleucine administered by mouth.

5. Normal animals became markedly anaemic when maintained on an isoleucine-deficient diet for more than a few weeks.

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An Improved Continuous Extractor for Liquids

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Prolonged heating of the extracted substances which occurs in many types of continuous extractor is a great disadvantage if any of the substances to be extracted are sensitive to heat. If, however, the extract can be removed and concentrated, losses caused by heating can be considerably reduced while the advantages of continuous extraction are retained.

In the apparatus here described this is achieved by passing the extract through a climbing-film evaporator where it is concentrated (in less than 1 sec.) and separated from the vapour in a cyclone. The solvent vapour is condensed and returned to the system to complete the cycle. A reservoir of fresh solvent is included to make up the volume as the concentrate is removed.

APPARATUS

Fig. 1 shows the general arrangement of the apparatus when used with solvents lighter than water. The condensed solvent vapour from the evaporator runs into the inner of two concentric cups in the control head *A*, the solvent flow for the extraction being controlled by the tap *B*, any excess overflowing into the 2 l. reservoir *E*. The solvent is then distributed in the 5 l. extractor *D* by the hollow turbine stirrer *C* as fine globules which rise to the surface to overflow into the reservoir *E*. By adjustment of the height of the reservoir and the tap *F*, the plug of which is cut to permit graduated opening, the mixed extract and excess solvent are led into the climbing-film evaporator *G* at a suitable rate. The evaporator consists of three parallel 36 × 0·25 in. tubes, constricted to 0·06 in. over a length of 0·5 in. at the bottom to prevent excessive surging. It is heated by a steam jacket. The extract is flash evaporated and the resulting spray passes tangentially into a cyclone *H* (made from a 500 ml. bolt-head flask) through a tube of cross-section area equal to the sum of the areas of the evaporator tubes. The centrifugal effect separates the concentrate from the vapour which is led out from the top of the cyclone to be condensed and so complete the cycle. The concentrate flows out from the bottom of the cyclone and is cooled and collected in the receiver.

The apparatus can readily be adapted to deal with solvents heavier than water.

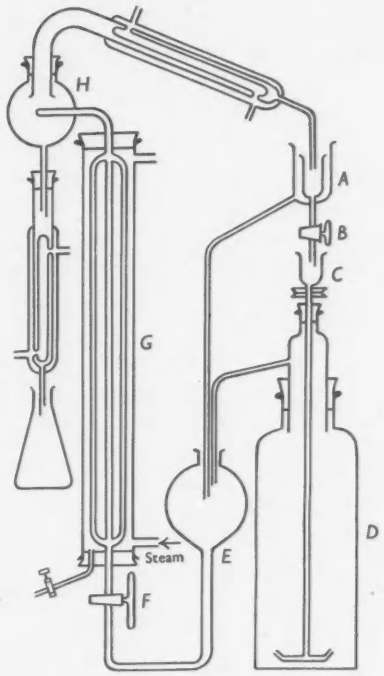

Fig. 1. Apparatus for extraction and concentration (not to scale). *A*, Control head; *B*, tap; *C*, hollow turbine stirrer; *D*, extractor; *E*, reservoir; *F*, tap; *G*, climbing-film evaporator; *H*, cyclone.

EXPERIMENTAL

0·02 % (w/v) Bromothymol blue solution (4·5 l.) in dil. HCl was placed in the extractor and 2 l. of benzene in the reservoir. The steam and water were turned on and the control tap *F* adjusted. A maximum distillation rate of 68 ml./min. was

obtained and the corresponding rate for the concentrate was 1·25 ml./min. giving a ratio of 54·3:1 and an estimated extraction time of 26·7 hr. before refilling of the reservoir becomes necessary.

When repeated with 4·57 l. of urine at pH 1·0 the extraction of 11-dehydro-17-hydroxycorticosterone and 17-hydroxycorticosterone was complete in the first period of 8 hr. as checked by chromatography (Zaffaroni, Burton & Keutmann, 1950).

SUMMARY

An apparatus is described for the extraction and concentration of heat-sensitive compounds.

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